

TECHNIQUES AND INSTRUMENTATION IN ANALYTICAL CHEMISTRY — VOLUME 16

FLOW-THROUGH (BIO)CHEMICAL SENSORS

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Preface

It is widely recognized that the great R&D endeavours expended on developing (bio)chemical sensors in the last few years have not yet met with the results demanded for extensive commercialization. The initial enthusiasm of the 1980s gradually gave way to cautious attitudes in the early 1990s and the current scepticism as regards the future. The best known authors in the field have started to acknowledge the gap between the major academic developments achieved so far and their applicability to real problems, which is the greatest current hindrance to extensive use of (bio)chemical sensors in routine analyses.

The primary purpose of this book is to draw the attention of the analytical chemical community in general, and those engaged in sensor development in particular, to the advantages of flow-through (bio)chemical sensors over classical probe-type sensors, the essential difference being in their external shape and operation. However small, such a difference is of great practical significance as it facilitates sample transport and conditioning, as well as calibration and sensor preparation, maintenance and regeneration, all of which result in enhanced analytical features and a broadened scope of application. Flow-through sensors are therefore more suitable for addressing real (non-academic) problems than are probe-type sensors. However, some of their well-known drawbacks, including poor selectivity, low precision and sensitivity, and a lack of durability, which are dependent on the particular ingredients involved in the recognition process, remain unsolved. However, the use of continuous configurations coupled on-line to flow-through sensors can help to indirectly improve the performance of many such sensors. This

is a promising technical approach to the implementation of (bio)chemical sensors, the success of which relies heavily on systematic investigations. This book presents the most salient advances in this context in a unique way compared to previously published monographs on the topic, some of which describe sensors according to the type of transducing system used, for example.

The introductory chapter provides an overview of (bio)chemical sensors and their impact on Analytical Chemistry. Essential concepts of flow-through (bio)chemical sensors including their definition, classification, the types of flow-cells where the sensing microzone can be accommodated, continuous-flow configurations to which they can be coupled, the measurement modes available and the types of transient signals obtained, among others, are the subject matter of Chapter 2. The other three chapters describe in an ordered way the most relevant types of flow-through (bio)chemical sensors, which are dealt with according to the processes taking place at the sensing (recognition) microzone, as well as their position in space and time. Thus, Chapter 3 is devoted to flow-through sensors based on integrated reaction and detection in the absence of a separation process; a distinction is made between sensors using an immobilized (bio)catalyst, antigen, antibody or chemical reagent. Flow-through sensors integrating a gas-liquid, liquid-liquid or solid-liquid separation with detection in the absence of a chemical reaction for recognition are discussed in Chapter 4, where special emphasis is placed on multi-determination sensors, ion-selective electrodes (ISEs) and ion-sensitive field-effect transistors (ISFETs) housed in flow-cells. Chapter 5 reviews flow-through sensors relying on the sequential or simultaneous integration of all three processes (separation, reaction and detection), which are grouped according to the type of separation (gas diffusion, dialysis, sorption) involved.

We should like to show our appreciation to those who have helped this book come to light. Thanks are due to the members of our research group (F. Lázaro, P. Linares, J. M. Fernández Romero, P. Richter and M. T. Tena) for the generous efforts that went into the development of new flow-through (bio)chemical sensors in our department; they were in fact the driving force for this project. We also wish to thank the staff of Elsevier Science Publishers for their warm welcome of our initial proposal. We are indebted to Antonio Losada, MSc, for translation and typesetting of the original Spanish manuscript, and Francisco Doctor and José M. Membrives for providing the artwork. Finally, we gratefully acknowledge financial support

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December 1993

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Sensors in Analytical Chemistry

1.1 ANALYTICAL CHEMISTRY AT THE TURN OF THE XXI CENTURY

Chemistry has three essential components, namely (a) theoretical principles, (b) synthesis and (c) analysis, the relationships between which are well known. The last of these three elements is unarguably the domain of Analytical Chemistry, however frequently it is "invaded" by other professionals who thus curtail analytical chemists' scope. From the above division of Chemistry into three fundamental parts an immediate, concise definition for Analytical Chemistry arises, namely the "science of chemical information", *i.e.* the science of chemical measurements. Traditionally, the term "metrology" has exclusively been assigned to physical measurements for no scientific reason. In fact, Analytical Chemistry can be defined in theoretical terms as the "chemical metrological science". In a meeting held in Edinburgh in 1993, the Working Party on Analytical Chemistry of the European Federation of Chemical Societies defined Analytical Chemistry as "a scientific discipline which develops and applies methods, instruments and strategies to obtain information on the composition and nature of matter in space and time". This definition encompasses both the basic and the applied connotations of this science, as well as the word "information", with which it is closely associated.

Current Analytical Chemistry can be considered to consist of three closely related parts, *viz.* (a) research and development; (b) the arsenal of techniques, methods and procedures formerly referred to jointly as "Chemical Analysis"; and (c) education [1]. Consequently, analytes and samples are no longer the targets of Analytical Chemistry; they have been superseded by the analytical problems derived from economic and social problems posed

by a world demanding ceaseless progress and changes. The major challenges of Analytical Chemistry at present can be summed up as the obtainment of more and better chemical information on any type of material or system of interest by using increasingly less material, time and human resources, and facing the lowest possible hazards [2].

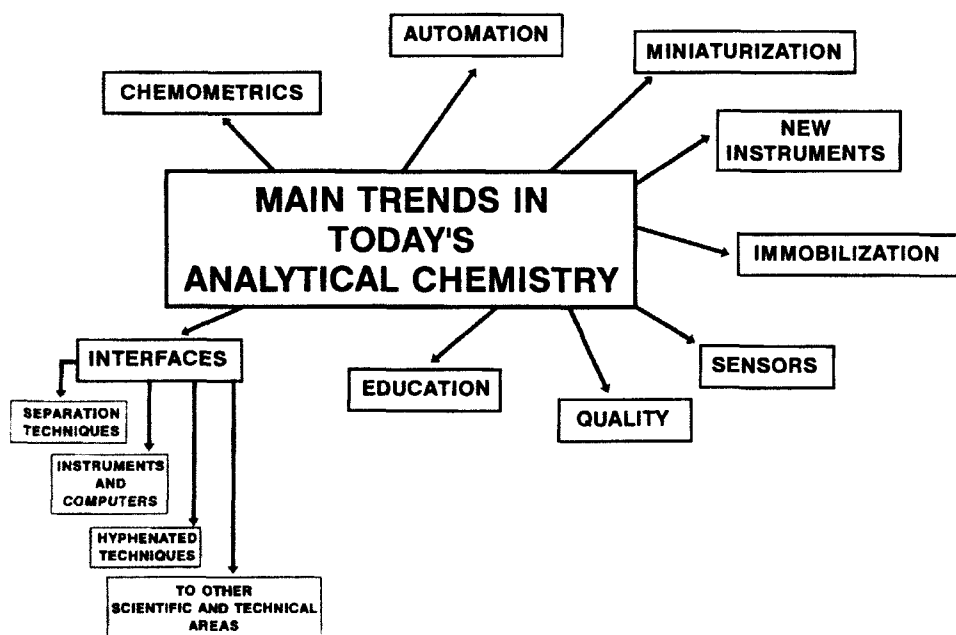


Figure 1.1 — Primary objectives of current Analytical Chemistry. (Reproduced from [1] with permission of Springer-Verlag).

Chemical information is pivotal to modern society and economy. Correct, well-founded decision making in economic and social affairs rests heavily on the quality of the information produced by analytical laboratories. Analytical chemists thus bear a high responsibility that entails expanding their activities beyond the laboratory walls (*e.g.* by undertaking process analysis, sampling, remote control or *in vivo* monitoring tasks), and establishing scientific and technical links with other professionals (physicians, engineers *etc.*) in order to play an active role in problem addressing and solving.

Figure 1.1. illustrates the most significant trends in Analytical Chemistry as regards the above basic objectives [1]. Some, such as automation and miniaturization (two essential keys to the development of new analytical instruments), are liable to integration. Chemometrics can also be regarded as a fertile ground for automation, and immobilization is essential to the development of new measurement approaches (instrumentation) and novel analytical separation techniques. Interfaces are of paramount significance to current Analytical Chemistry. They can be of purely physico-chemical nature (*e.g.* in separation techniques); electronic (between instruments and computers, which can be linked in an active or passive way); between techniques; and interdisciplinary (with other scientific and technical areas). The development of reliable (bio)chemical sensors is no doubt rather an interesting goal as these devices enable implementation of highly enhanced analytical processes. Of special note in this context is the growing significance of quality approaches to the analytical laboratory [3]. As far as education is concerned, Analytical Chemistry tutors should at any rate narrow the gap between what they teach and what is actually being developed (research) and used at present [1].

1.2 ANALYTICAL INFORMATION

There are two types of analytical information as regards its target and origin. *External* analytical information originates from a bidirectional relationship between the analytical chemist and (a) society, (b) the body to which the laboratory is answerable, (c) other scientific and technical areas, (d) literature sources (which the analytical chemist uses and expands), and (e) students. On a lower level, subordinate to external information, *internal* information originates from a uni- or bidirectional relationship between the analytical chemist and (a) instruments (and apparatuses) and (b) computers, in addition to the information transferred between interfaced instruments and between computers and instruments.

Several conceptual and technical orderings based on the analytical information level can also be established (see Fig. 1.2). Reports contain information of the highest level; in fact, in addition to the results, they provide an interpretation that is facilitated by chemometric techniques (*e.g.* those based on pattern recognition) and cooperation with other scientific and technical areas. In fact, reports provide answers to the problems addressed. Results are referred to samples and analytes, and are arrived at by chemo-

metric treatment of raw data (*e.g.* potentials, fluorescence intensities, absorbances). There are also secondary data (*e.g.* temperatures, flow-rates, pressures, rotation speeds) associated with the operation of apparatuses and instruments which influence the quality of raw data but are not directly related to the results [4].

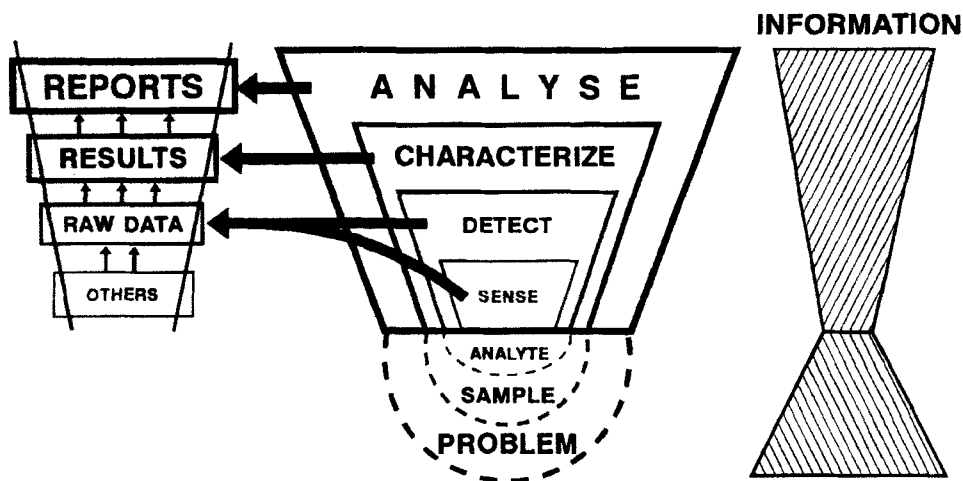


Figure 1.2 — Technical hierarchies of analytical information. For details, see text.

One hierarchy of great relevance to the contents of this book is that which arises from information contents between "to sense" and "to analyse" (Fig. 1.2). *To sense* entails using a device responding to the concentration or presence of a given chemical or biochemical species in a sample. *To detect* involves using an instrument to input and/or output a signal, whether induced or not, and transduce it to a readily measurable quantity. Both terms refer to the analyte(s) and give rise to the so-called "raw data". *To characterize* is to identify differential features of the sample or system studied in order to obtain analytical results. Finally, *to analyse* involves characterizing and interpreting in qualitative, quantitative or structural terms a sample or system in order to issue an analytical report in response to an analytical problem arising from another (social or economic) problem. Thus, an electrochemical sensor accommodating an immobilized catalyst (*e.g.* in the working electrode) responds to the presence or concentration of a given analyte; the sensor can be connected to a voltammetric detector (an instru-

ment) for measuring the current intensity (a signal), a primary datum that can be used to fully characterize the sample in question —usually by employing additional sensors and detectors.

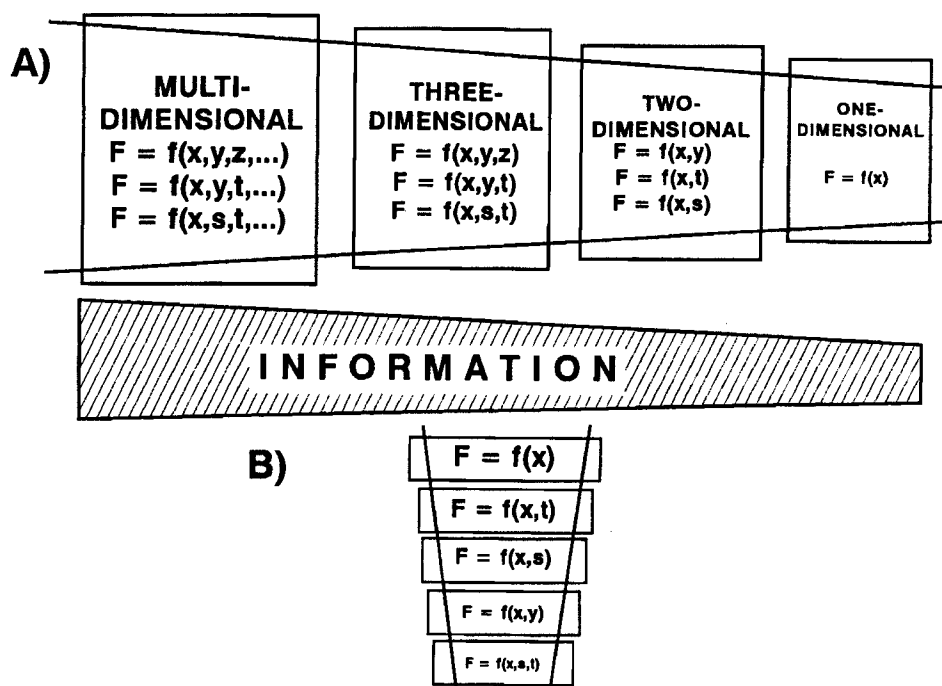


Figure 1.3 — (A) Dimensionality of analytical information. (B) Frequency ordering of the different types of information produced (and used) in connection with sensors.

Figure 1.3.A shows the scheme for another analytical information hierarchy that is complementary to the previous ones. Thus, gravimetries, titrimetries, classical qualitative analyses and sensors provide one-dimensional information of the form $F = f(x)$, where x is the signal concerned. On the other hand, instrumental techniques provide two-dimensional information that can be of two types depending on whether the signal (x) is combined with an instrumental parameter (y), time (t) or space (s). Some modern analytical techniques (several of which use hybrid instruments) furnish three-dimensional information by combining signals with one or two instrumental parameters (y, z), time and space. The great

processing power of computers will foreseeably allow multi-dimensional information on the analytical process to be obtained in the very near future. Figure 1.3.B shows an ordering of the type of information produced by (bio)chemical sensors according to frequency of use. This hierarchy is discussed at length later on.

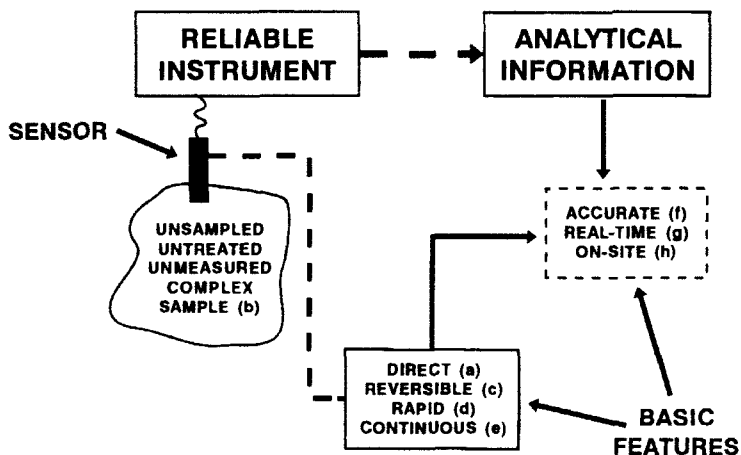


Figure 1.4 — Features of the ideal (bio)chemical sensor.

1.3 WHAT IS A SENSOR?

Defining the word "sensor" is far from easy. Because a sensor is currently regarded by many as the magic key to a number of doors, the term is very often used improperly. No doubt, the personal bias of the many authors who have provided a definition for "sensor" has fostered the wide variety of existing interpretations. Thus, some consider a sensor to be "a wavelength" at which the absorbance of an analyte or reaction product is measured. Others have an even vaguer idea and call an FIA assembly a sensor, for example.

There are two complementary definitions of sensor based on its operation—use and composition (structure). Thus, a sensor can be defined as a miniature analytical device (not an instrument or an apparatus) that responds to the presence and/or concentration (activity) of an analyte—species and, according to Fig. 1.4:

(A) Is brought into *direct contact* (a) with the uncollected, unmeasured, untreated sample (b);

(B) Operates *reversibly* (c) —it responds equally to low and high analyte concentrations— *rapidly* (d) and *continuously* (e); and produces a signal via a reliable instrument which provides analytical information that is:

(C) *accurate* (f) (i.e. highly selective, sensitive and precise);

(D) obtained in *real* or *near-real time* (g), and

(E) *on site* (h).

Figure 1.4 shows a schematic diagram for the ideal (bio)chemical sensor (one meeting the above requisites as regards the device itself and the analytical information it produces, both of which are obviously mutually related).

Based on its structure, a sensor is an analytical device consisting of two main parts: a (bio)chemical microzone (recognition element) that is brought into contact with the sample and closely associated (connected or integrated)

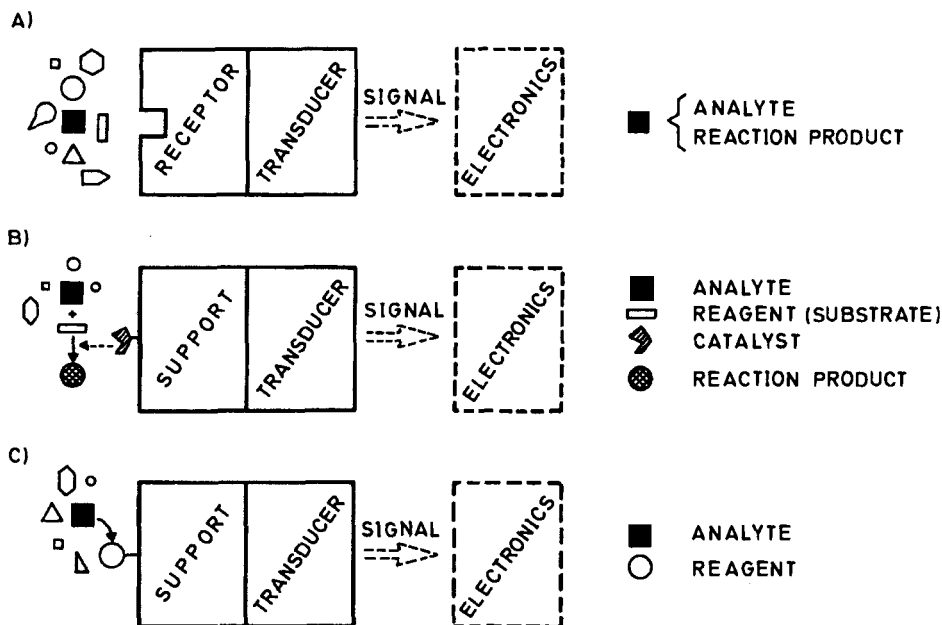


Figure 1.5 — Basic types of mechanism involved in the sensing process. For details, see text.

with an optical, electrical, thermal, mass or acoustic transducer for conversion of the recognition pattern into a (usually electric or optical) primary signal that can be amplified and measured by means of a detector (instrument). The measurement is suitably transformed and the results expressed as a pH, molarity, amount of substance (*e.g.* grams per weight or volume unit), pressure (torr, kPa), *etc.*, by using a data acquisition and processing system. Figure 1.5 shows three general schemes for the sensing process that can take place in the recognition element (the one that receives or supports an immobilized species), namely: (a) specific retention of the analyte (*e.g.* the typical lock-and-key mechanism of immunological sensors); (b) a (bio)chemical reaction catalysed by a species retained on the support (the reaction product is thus the subject of the recognition process); and (c) a solid-phase reaction inducing a change in a reagent (indicator) retained on the support. Some other alternatives also exist and are discussed in this book.

Figure 1.6 shows the major types of recognition elements and transducing systems used for implementing (bio)chemical sensing. A detailed discussion is provided in other sections of this chapter and the selected examples described throughout this book. Recently, Wolfbeis reported a systematic review of recognition elements based on enzymes, ion-carriers and molecular interactions used in optical sensors [5].

The two definitions above can be used as the starting point for establishing the technical and operational features of the ideal (bio)chemical sensor. However, the word sensor is applied in practice to devices not meeting all the requisites but sharing some common characteristics including (a) their being devices rather than instruments, (b) their small size and portability; (c) the fact that they integrate detection with a separation and/or (bio)chemical reaction at the same microzone; and (d) their being brought into more or less close contact with the sample. Thus, some devices not working in a fully reversible manner (*e.g.* disposable or single-use and irreversible reusable devices), and others not operating in a fully continuous fashion [or even lacking an active microzone giving rise to the signal, as is the case with passive fibre-optic near infrared (NIR) sensors], are typically regarded as sensors. As noted later, many reported sensors lack the sensitivity, selectivity and precision required to ensure accurate results. Very often, a (near) real time or on-site response is of little value. Therefore, the above twofold definition of sensor can be taken as both a guideline and an

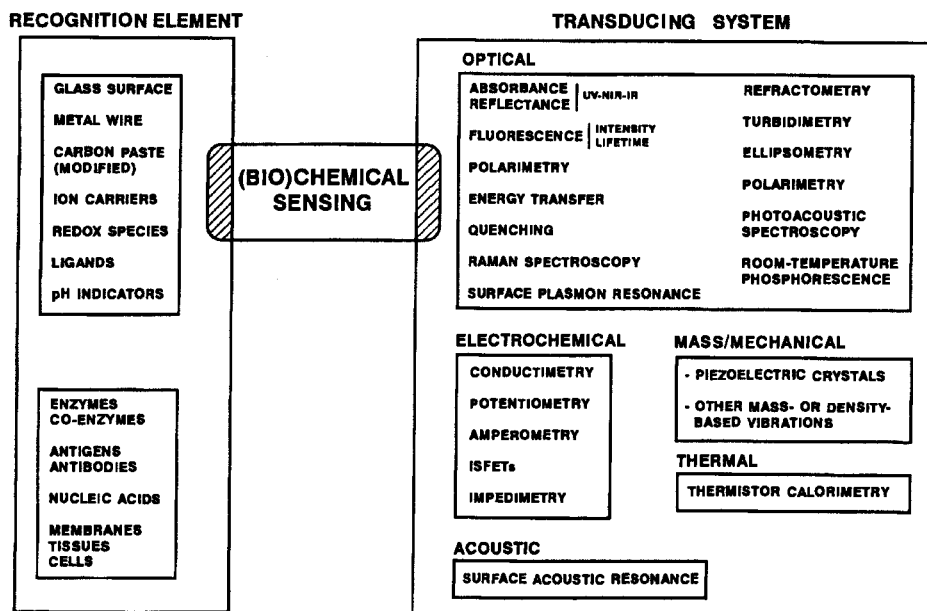


Figure 1.6 — Elements of a (bio)chemical sensor. (Adapted from [5] with permission of Elsevier Science Publishers).

ideal proposition. The literature on sensors in general and this book in particular describe sensors that deviate markedly from the basic guidelines exposed. However, they bear some resemblance to the above-described ideal sensor and are clearly different from other analytical devices also providing analytical information (*e.g.* diode array detectors); they are obviously different from apparatuses, which perform a function but provide no analytical information, as well as from conventional instruments. Obviously, some analytical approaches could also be considered sensors (*e.g.* devices used for analysis of solids by laser ablation atomic spectrometry, LAAS [6]) as they meet some of the above requisites; in any case, they do not fulfill the four common, minimal requirements — LAAS devices are far from miniature-size, for example.

1.4 SENSORS AND THE ANALYTICAL PROCESS

The evolution of Analytical Chemistry in time has not been too harmonious. The analytical process, *viz.* the set of operations performed in order to obtain the desired results from a raw (uncollected, untreated, unmeasured) sample, is typically executed in three steps, namely: (*a*) preliminary operations (sampling, sample preservation and treatment, subjection to separation techniques); (*b*) measurement and transducing of the analytical signal, which entails using an instrument; and (*c*) data acquisition and processing. The last two have reached a stage of development that could hardly be envisaged not long ago. The quality of currently available instrumentation is unquestionable, as is the affordability and processing power of (micro)computers and chemometric software. While growth in this field is still possible, genuinely innovative ideas do not emerge so easily, so further, worthy developments are few and far between. On the other hand, advances in preliminary operations have run at a much slower pace than at the other two stages of the analytical process despite their doubtless, decisive significance for obtaining quality analytical information in an expeditious, economical, and human and environmentally safe way. However good the instrumentation and chemometric software currently available may be, the results can never be of adequate quality if preliminary operations are not properly performed [7].

Preliminary operations are rather variable in nature, labour-intensive and time-consuming (or even tedious) and the source of major bias and accidental errors that may have a decisive influence on the quality of the analytical results. In addition, they escape systematic control, which is in clear contrast with the ease with which an instrument can be calibrated, and are a major source of potential hazards to both laboratory staff and the environment. These adverse characteristics make preliminary operations a pending matter for Analytical Chemistry [7] that demands generous R&D endeavours for improvement. Some of the most salient recent advances in this context include the development of robot stations [8,9]; the increasing use of non-chromatographic continuous separation techniques [10]; the development of automatic sample pretreatment modules using conventional, ultrasonic [11] or microwave energy sources [12]; downscaling of existing industrial and preparative chemistry innovations including lyophilization [13] and supercritical fluid extraction [14,15]; the development of process analysers [16–18]; and the use of (bio)chemical sensors, the subject matter of this book.

What is the underlying reason for the unusually great interest that sensors have aroused in Analytical Chemistry? The answer is utterly simple. In fact, they allow implementation of the first two stages of the analytical process, as shown in Fig. 1.7. A sensor complying strictly with the definition given in Fig. 1.4 will obviously be an ideal choice for circumventing many of the pitfalls of preliminary operations (e.g. those arising from complex samples, sluggishness, intensive operator involvement, accidental errors, hazards) but not others such as those originating from sample variability or factors escaping control. In addition, despite a few worthy attempts, sensors are mostly incompatible with solid samples, even though the associated difficulties can be overcome by using a suitable separation technique in on-line combination with the sensor (the joint use of supercritical fluid extraction and flow-through sensors may open new, interesting avenues in this context [19]).

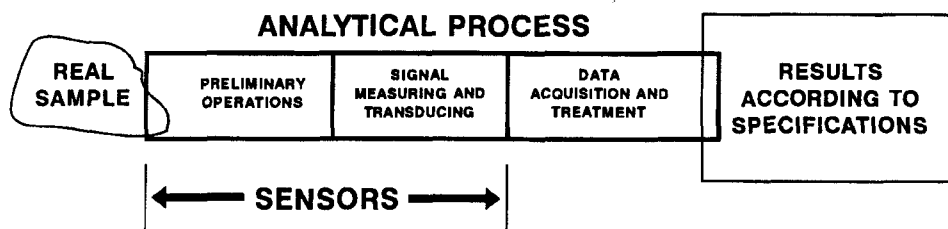


Figure 1.7 — Significance of sensors to the analytical process.

Broadly speaking, sensors introduce three different technical assets in the analytical process that are consistent with the scientific and technical trends of the end of this century. They are schematically presented in Fig. 1.8 and commented on below. First, sensors reduce human participation in preliminary operations as they facilitate automation of the analytical process stage in greatest need of it [20,21]. Also, the inherently small (miniature) size of sensors makes them usable for such novel interesting applications as *in situ* monitoring in general and *in vivo* monitoring in particular. The third major advantage of sensors is no doubt extremely important—though occasionally undervalued—, namely simplification, particularly as regards sub-steps of preliminary operations. These three technical advantages are mutually related.

Thus, simplification clearly facilitates automation and miniaturization, and miniaturization assists automation.

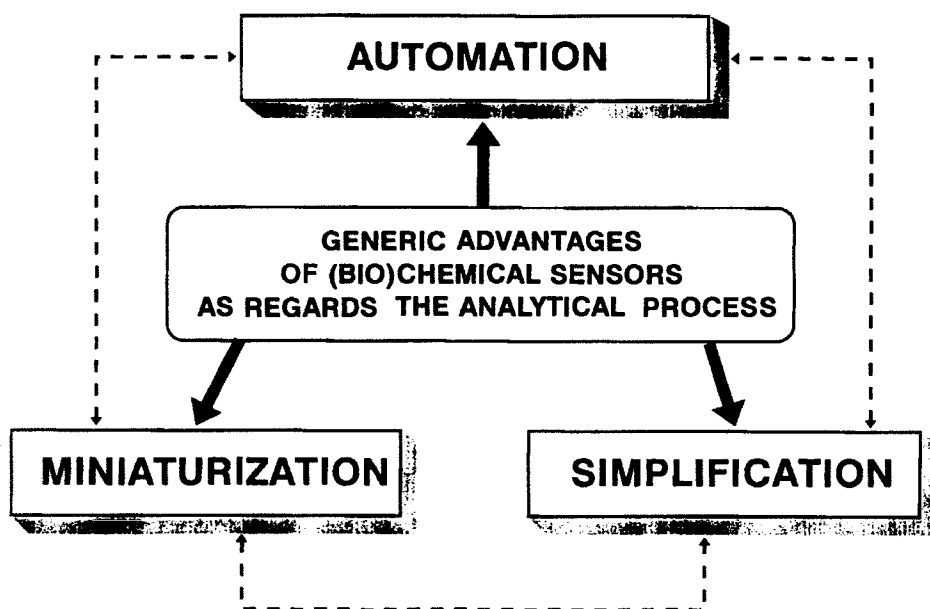


Figure 1.8 — Principal technical assets of sensors as used in Analytical Chemistry.

No wonder sensors are becoming increasingly significant to the analytical science. Other than their use in the laboratory, applications in non-analytical tasks are probably the greatest driving forces for research and development in this area. No doubt, the need for continuous monitoring of critical parameters in such areas as clinical chemistry; biotechnology; the pharmaceutical, chemical, nuclear and chemical warfare proliferation control; and environmental chemistry can be reliably met by using sensors. The role of sensors in in-line process monitoring (direct introduction of the sensor into the evolving system) is complementary to their growing use in on-line continuous systems as substitutes for traditional detectors (instruments). Figure 1.9 illustrates the relationship between sensors and process monitoring approaches. It is interesting to note that automatability and simplification increase and the ease with which reliable calibration can be achieved

decreases from the off-line to the non-invasive approach. As a result, the advantages inherent in the use of sensors (Fig. 1.8) are countered by the difficulty involved in accomplishing secure, efficient calibration. This shortcoming is circumvented by mixed on-line/in-line systems, which are thus advantageous in this respect. This is one of the favourable features of the flow-through sensors described in this book: they essentially possess the assets of sensors and enable ready, dependable calibration [22].

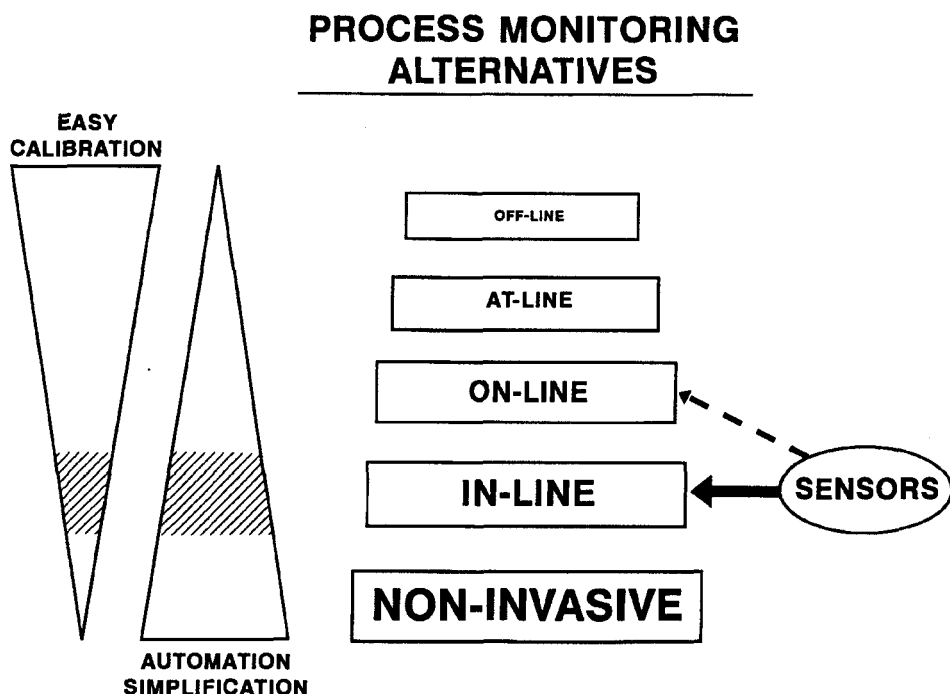


Figure 1.9 — Use of sensors in process monitoring.

Any powerful technology has two faces. In fact, the adverse connotations of nuclear power (*e.g.* atomic bombs, radioactive pollution) are offset by important positive applications (*e.g.* in nuclear medicine). Sensors have two opposing faces as well. On the one hand, they make a major choice for the Analytical Chemistry of the XXI century; on the other, the ease with which they can output signals (and hence results) may induce careless (non-contrasted) use and obtainment of spurious analytical information, particularly in the hand of non-experts (*e.g.* physicians, engineers) obviously lacking

the Analytical Chemistry background required for reliable handling of these devices. These non-analytical professionals regard sensors as the analytical ideal, a goal to be reached, but never as a potential mirage. Consequently, sensor marketing should run in parallel with the development of calibration systems ensuring a minimum quality.

1.5 TYPES OF SENSORS

The impact of sensors on Analytical Chemistry has fostered both basic developments and publication of an exceedingly large number of literature reviews (*e.g.* [23–34]) and monographs (*e.g.* [35–49]). Close examination of such an abundant bibliography on the topic (over a thousand papers per year in the 1990s [23]) reveals the wide variety of sensors available, but also the need to set up some conceptual and technical orderings so as to assist novices in the topic. This section presents several classifications of sensors according to various criteria; such classifications are complementary, but never dogmatic or inflexible (other potential alternatives might indeed have been included and some of those that are lie in between two of the groups of proposed classifications). Figure 1.10 presents several such classifications for (bio)chemical sensors; physical sensors, primarily intended for monitoring a physical quantity such as temperature, pressure or viscosity, are excluded.

One ordering of (bio)chemical sensors is based on whether the analyte (and, occasionally, the sample) is of a chemical or biochemical nature, and is thus in apparent contradiction with another classification based on the nature of the recognition element. Thus, a sensor using an immobilized enzyme for determining an analyte of biological interest (*e.g.* glucose) can be considered a biosensor. On the other hand, if it uses a chromogenic ligand for determining a metal ion it can be regarded as a chemical sensor. However, one that employs a biological material for determining a chemical substrate (or an inhibitor) in a biological sample may in principle be considered a chemical or biochemical sensor, even though there is a trend to designating it by the latter denomination.

(Bio)chemical sensors can be active or passive according to whether they use a sensing microzone to accommodate a chemical or biochemical reaction and/or a biochemical (*e.g.* immunological) or physico–chemical separation (*e.g.* sorption). It should be noted that passive sensors (*e.g.* a fibre-optic tip immersed in an industrial process stream) do not meet one of the essential requirements included in the definition of sensors as regards composition

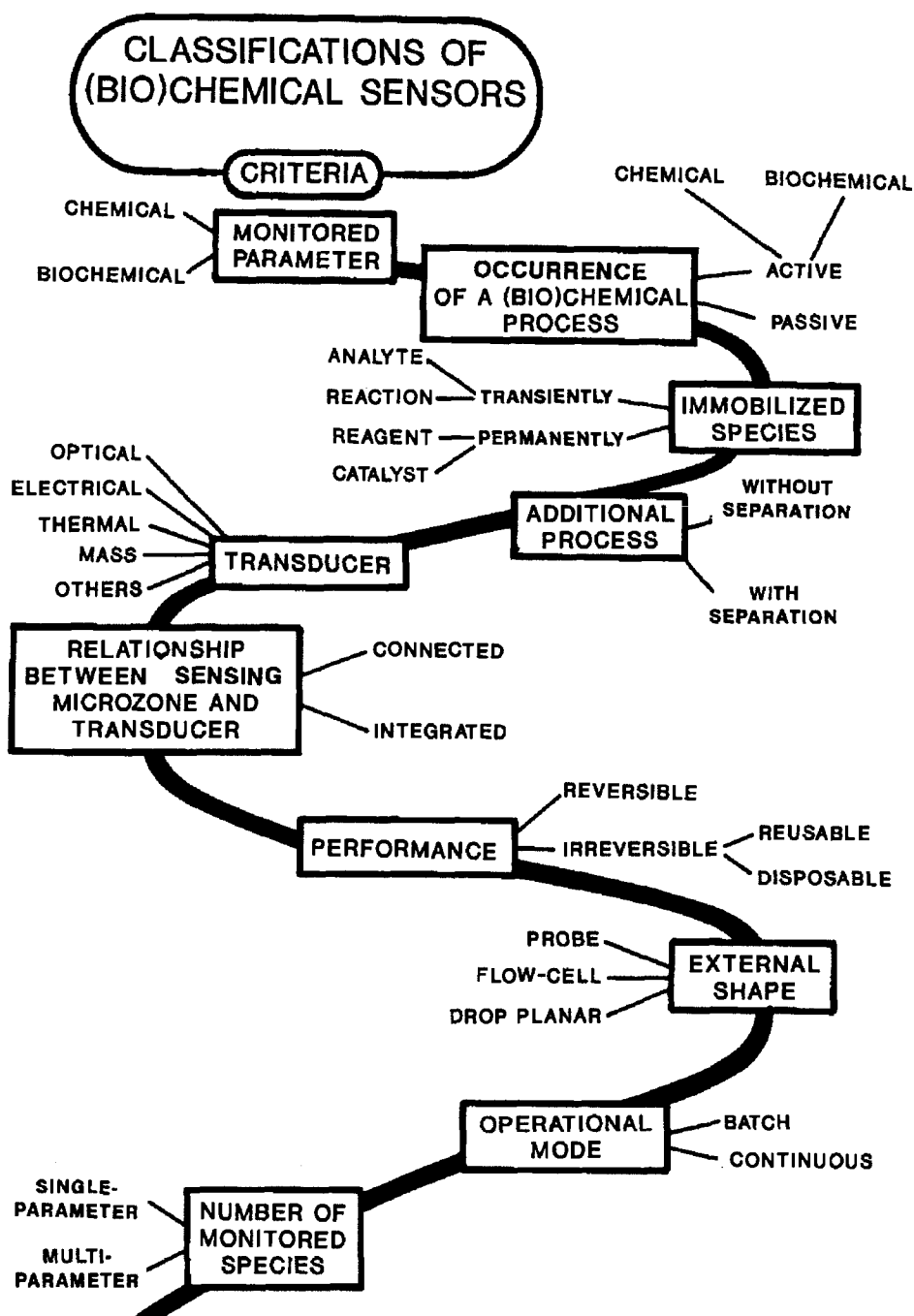


Figure 1.10 — Classifications of (bio)chemical sensors based on various criteria.

(*viz.* existence of a sensing microzone) as they rely on direct measurements of a physico-chemical property of the analyte; however, they are operationally similar to active sensors and have the added advantage of full reversibility.

(Bio)chemical sensors can also be classified according to which species is immobilized at the sensing microzone. Such a species can be any of the ingredients of a (bio)chemical reaction, *viz.* the analyte, reagent, catalyst or product (one of them if there are several). In addition, the species in question can be immobilized permanently if it is the reagent or catalyst, or temporarily if it is the analyte or a reaction product. Some sensors involve twofold immobilization: they use the reagent in permanently immobilized form and retain the analyte at a later stage, or employ two permanently immobilized species (*e.g.* the reagent and catalyst). A detailed description of these and other variants is provided in Chapter 2.

There are some (bio)chemical sensors where the recognition process by which the response is produced involves no implicit mass transfer. Such is the case with sensors using catalysts (enzymes) immobilized in the sensing microzone, where only a (bio)chemical reaction takes place (Fig. 1.5.B), and sensors based on an immobilized reagent that undergoes a change on interaction with the analyte, which is not retained (Fig. 1.5.C). Many sensors rely on a separation process that takes place at the recognition microzone. The process is usually one of sorption (*e.g.* see Fig. 1.5.A) and simultaneous with detection; alternatively, a gas-diffusion, dialysis or liquid-liquid extraction separation can be used sequentially or simultaneously with detection, in the presence or absence of a (bio)chemical reaction. In many cases, separation cannot be distinguished from detection (*e.g.* in immunosensors based on a lock-and-key mechanism).

The sensors discussed in this book are dealt with according to the classifications based on which the immobilized species is, the type of immobilization used and whether or not an additional separation is included. Thus, sensors have been included in three chapters according to the type of process that takes place in their sensing microzone, namely reaction-detection (Chapter 3), separation-detection (Chapter 4) and separation-reaction-detection (Chapter 5).

No doubt, one of the most intuitive classifications of sensors is that based on the type of transducer used to reveal the physico-chemical changes that occur in the sensing microzone in the presence of the analyte. Figure 1.6 shows the principal types of transducing systems are connected to or

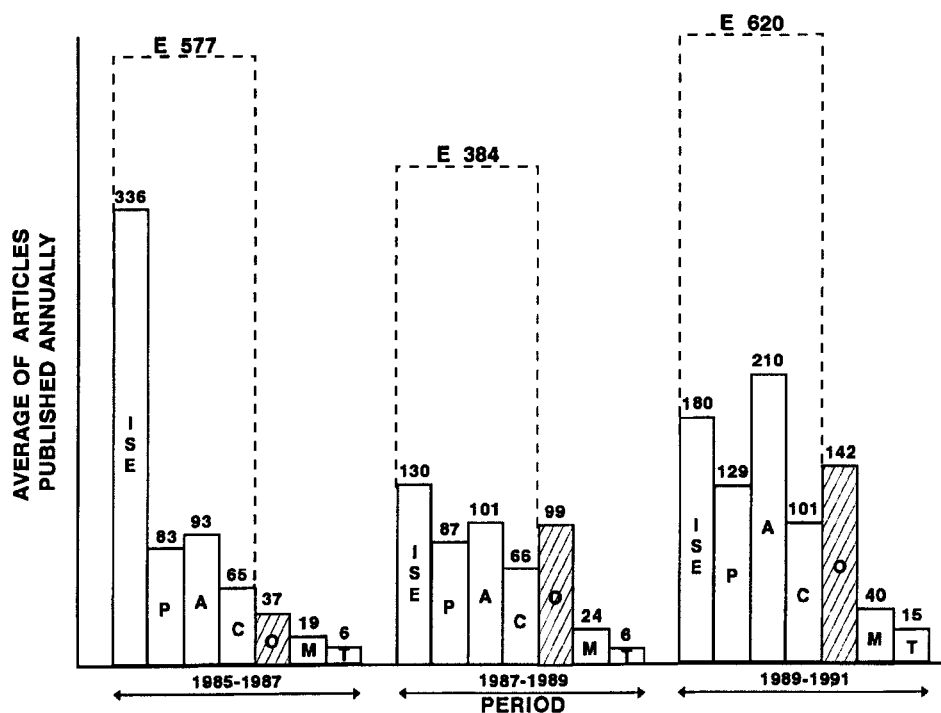


Figure 1.11 — Average number of papers on (bio)chemical sensors published annually, based on data from Janata's biannual review. E electrochemical sensors; ISEs ion-selective electrodes; P potentiometric sensors; A amperometric sensors; C conductimetric sensors; O optical sensors; M mass sensors; T thermal sensors. (Adapted from [23] with permission of the American Chemical Society).

integrated with the sensing microzone. Historically, the pH electrode and ion-selective electrodes (ISEs) in general were the earliest to be developed and continue to arouse—gradually decreasing—research and development interest. Optical, thermal and mass sensors have gained enormous momentum in the last few years, whereas electrochemical (potentiometric, conductimetric and amperometric) sensors reported in the same interval have levelled off in number. Figure 1.11 shows in graphical form the evolution of sensors; the graph is based on data from Janata's biannual review [23], which is not exhaustive as the author himself admits.

One very useful technical classification of sensors establishes two categories according to the relationship between the recognition element and the transducer. These two essential elements can be connected optically (*e.g.*

by use of fibre optics) or electrically. In addition, the transducer (detector) and the sensing microzone can be integrated in a single element. A detailed description of these two options is given in Section 2.2 (see Fig. 2.6).

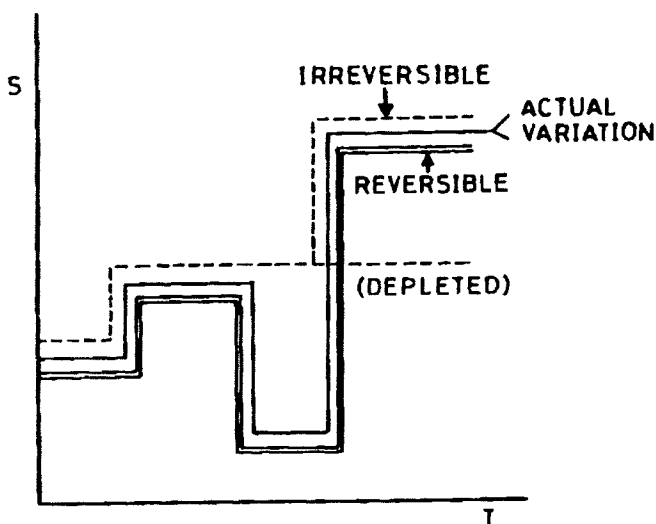


Figure 1.12 — Comparison of the response of reversible and irreversible–non-regenerable sensors with the actual signal variation. S signal; t time. For details, see text. (Reproduced from [21] with permission of VCH Publishers).

The ideal (bio)chemical sensor should operate reversibly and respond like a physical sensor (*e.g.* a thermometer), *i.e.* it should be responsive to both high and low analyte concentrations and provide a nil response in its absence. One typical example is the pH electrode. In short, a reversible (bio)chemical sensor provides a response consistent with the actual variation in the analyte concentration in the sample and is not limited by any change or disruption; in practical terms, responsiveness is inherent in reversibility. An irreversible–non-regenerable (bio)chemical sensor only responds to increases in the analyte concentration and can readily become saturated; only those (bio)chemical sensors of this type intended for a single service (disposable or single-use sensors) are of practical interest. On the other hand, an irreversible–reusable sensor produces a response similar to that from an irreversible sensor but does not work in a continuous fashion as it requires two steps (measurement and renewal) to be rendered reusable. Figures 1.12 and 1.13 show the typical responses provided by this type of sensor. Note

that the response of a reversible sensor coincides with the actual variation of the signal and that this behaviour is similar to that of irreversible–reusable sensors provided they are subjected to the two above-mentioned steps. Irreversible–non-regenerable sensors give rise to no signal increases or decreases and can readily become saturated (*i.e.* the signal can reach a constant level whatever the analyte concentration present [21]).

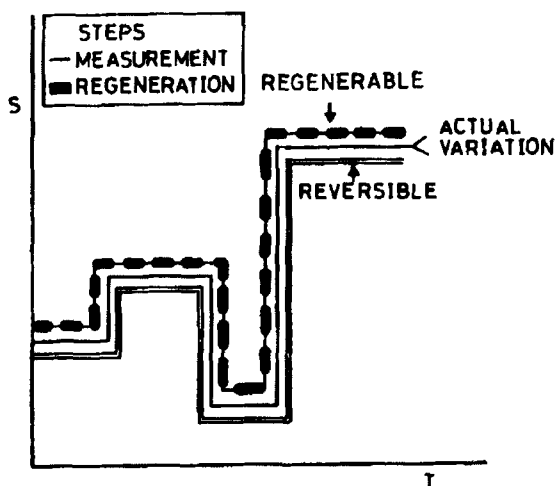


Figure 1.13 — Comparison of the response of irreversible–regenerable sensors with the actual signal variation. *S* signal; *t* time. For details, see text. (Reproduced from [21] with permission of VCH Publishers).

One crucial classification for this book is that based on the sensor external shape and the way the sensing microzone (SMZ) is brought into contact with the sample. Figure 1.14 shows a pertinent schematic diagram. The sensing microzone in a probe-type (bio)chemical sensor is located at the tip of a probe (*e.g.* an electrode, an optical fibre) and is plunged into the sample; the recognition element is connected to the transducer (detector). It is interesting to note the difference between this conception and that supported by other authors (*e.g.* see page 2 in reference 37, vol. I), who use the word "probe" to designate irreversible disposable sensors, yet admit that the borderline between sensors and probes is vanishing. In this book, the word "probe" is used in its widest acceptance (a small, rod-shaped object that is used to examine a system), which is also consistent with the definition of Webster's New International Dictionary, namely "something usually

pointed and slender that resembles or is suggestive of a surgical instrument and is used to penetrate, poke or prod in an exploratory way". The sensing element in a drop planar sensor is a flat microzone onto which sample drops impinge. This type of sensor is usually disposable. Typical examples include pen-type electrochemical sensors for *in situ* monitoring of the glucose level in whole blood for daily use by diabetics —each sensing element is used only once. Dosimeters are also planar sensors designed for inundative assays; they provide no real-time data and are not reversible. Finally, in flow-through sensors (the subject matter of this book), the sample is aspirated (or injected) and transferred to a flow-cell accommodating the sensing microzone, which can be integrated with or connected to the transducer (see Fig. 2.6 for details); the sample (carrier) stream can be driven to waste or returned to the sample flow. A more detailed overview of the differences between probe-type and flow-through sensors is provided in Chapter 2 (e.g. see Figs 2.3 and 2.5).

(Bio)chemical sensors can be used in both the batch and the continuous mode. While this is also true of probe-type sensors, flow-through sensors can only be used in a continuous regime coupled on-line to a continuous-flow configuration.

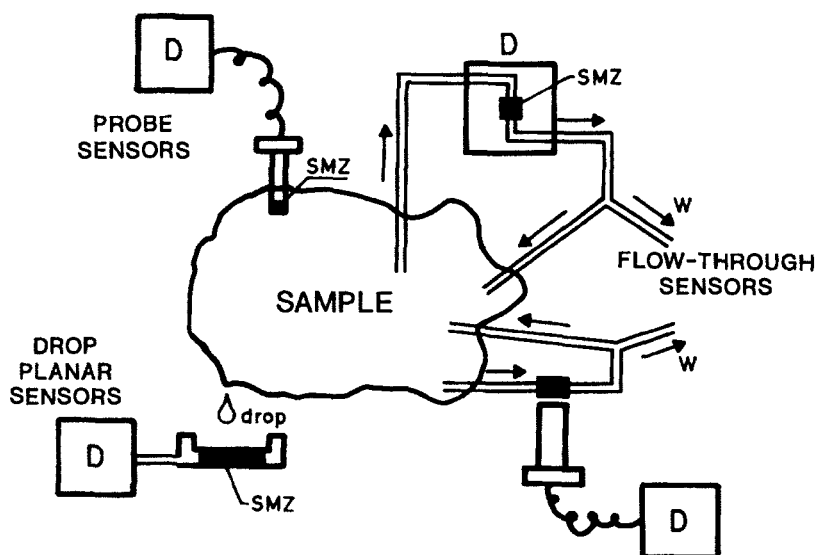


Figure 1.14 — Classification of flow-through sensors according to external shape. SMZ sensing microzone; D detector; W waste. For details, see text.

Finally, one other interesting classification categorizes (bio)chemical sensors according to the number of analytes that can be monitored in the same sample. Most sensors respond to the concentration of a single analyte. These single-parameter sensors can be of two types, *viz.* (a) those that are sensitive to a single analyte and whose sensing microzone houses highly selective processes (reactions and/or separations); and (b) those that respond to a family of chemical compounds (*e.g.* P-pesticides), which are highly useful for overall screening determinations (*e.g.* to obtain yes/no answers). There are two basic types of multi-parameter sensors, namely (a) those using several recognition elements (one per analyte) in the same sensing microzone; (b) and those using a single recognition element connected to or integrated with a transducer featuring multi-signal handling capabilities (*e.g.* a diode-array photometric detector). In the latter case, each analyte must give rise to a resolvable signal (*e.g.* a distinct molecular absorption spectrum) and the multi-signal acquired must be processed by using a chemometric approach.

1.6 GENERAL FEATURES OF (BIO)CHEMICAL SENSORS

The twofold definition given in Section 1.3 encompasses several properties a (bio)chemical sensor must have in order to fulfil the objectives demanded by the analytical quality level to be achieved. Some such properties are mandatory, whereas others are only desirable. Figure 1.15 shows advantageous and essential sensor properties arranged in four groups that are discussed below.

The first group of sensor properties in Fig. 1.15 is concerned with the quality of results obtained in analytical processes involving a (bio)chemical sensor. All of them are obvious targets of analytical tasks [3]. As shown in the following section, the accuracy of the analytical results relies on a high reproducibility or repeatability, a steep slope of the calibration curve (or a low detection or quantification limit) and the absence of physical, chemical and physico-chemical interferences from the sample matrix. Sensors should ideally meet these essential requisites. Otherwise, they should be discarded for routine analytical use however great their academic interest may be.

The second group of properties is concerned with sensor operation in general and the peculiarities of the recognition element in particular. Ideally, a sensor should be fully reversible. Otherwise, it should lend itself readily to rapid, effective, reliable regeneration in order to be actually reusable. If

the recognition element is irreversibly altered during the sensing process and hence the sensor cannot be regenerated, it will only be of practical use if its performance (*e.g.* price, portability, suitability for solving a complex analytical problem) offsets the expenses involved in using a new (disposable or single-use) sensor each time.

The temporal features of (bio)chemical sensors are vital but frequently overlooked in developing new methodological approaches, either because their significance is poorly known or because such features are not good enough. Thus, a (near) real time response is essential if a sensor is to be used for on-line process monitoring. Accordingly, the processes taking place at the sensing microzone (a biochemical reaction and/or a separation) should be as rapid as possible (not longer than a few seconds), not only when the

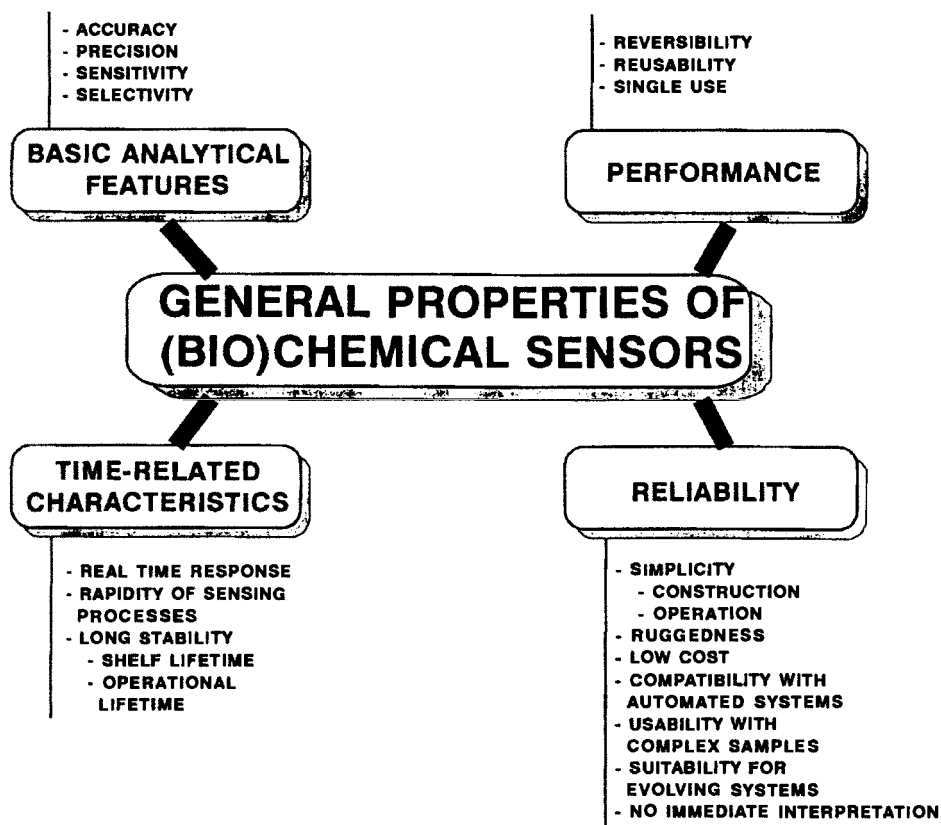


Figure 1.15 — Essential and desirable (bio)chemical sensor properties.

microzone is brought into contact with the analyte, but also when its concentration changes for some reason. A rapid initial response is not sufficient for a reversible sensor; in fact, the signal should fall to its original level (e.g. zero) within a few seconds in the absence of analyte. Irreversible-reusable sensors should feature both a rapid initial response and expeditious regeneration. Sensor durability is one other factor of great practical significance that can be measured as a shelf lifetime (*viz.* the time elapsed between construction and use) and a service lifetime (*viz.* the length of time during which the sensor can be used in routine applications or the number of samples it can process with no degradation in its performance or response). These last two factors have so far severely restricted marketing of (bio)chemical sensors (particularly those using immobilized biochemical species). Ample endeavours are still required in order to develop sensors with reasonably long shelf and service lives. Technical approaches to the preservation of the sensing microzone are of special significance in this respect.

Other interesting properties of (bio)chemical sensors are related to their effectiveness for solving real analytical problems. First, these analytical devices should be easy to construct, operate and preserve. Ruggedness, defined here as the confidence that small variations in the experimental conditions (pH, temperature, ionic strength, pressure) will not alter the sensor functioning or response, is very important. A low cost is also desirable, particularly with single-use (bio)chemical sensors.

Compatibility between sensors and automatic and automated analytical systems is crucial as it allows two Analytical Chemistry trends to be combined (see Fig. 1.1). Probe-type and planar sensors can be used in automated batch systems including robot stations, as well as in continuous (mixed in-line/on-line) systems. On the other hand, flow-through sensors are only compatible with continuous configurations.

Based on the definition for the ideal (bio)sensor, one such sensor should be directly applicable to a complex sample with minimal pretreatment, even though some conditioning (pH adjustment, addition of a masking agent) is involved in dealing with liquid samples, which is most conveniently done if the sensor is coupled on-line to an automatic continuous configuration, which in turn facilitates implementation of routine steps including calibration, reparation and storage. Gaseous samples are rarely subjected to any pretreatment owing to the intrinsic difficulties involved; however, the sensor to be used can be immersed in an analyte recipient solution through which

the gas is bubbled. It should be noted that solid samples are generally incompatible with conventional (bio)chemical sensors —there are some instances of direct application of sensors to a solid unknown, though. This pitfall can be circumvented by using a continuous leaching technique (*e.g.* SFE) coupled on-line to a (bio)chemical sensor for direct monitoring of the collecting zone (a solution, cryogenic trap or active solid) over which the depressurized supercritical fluid is passed [19].

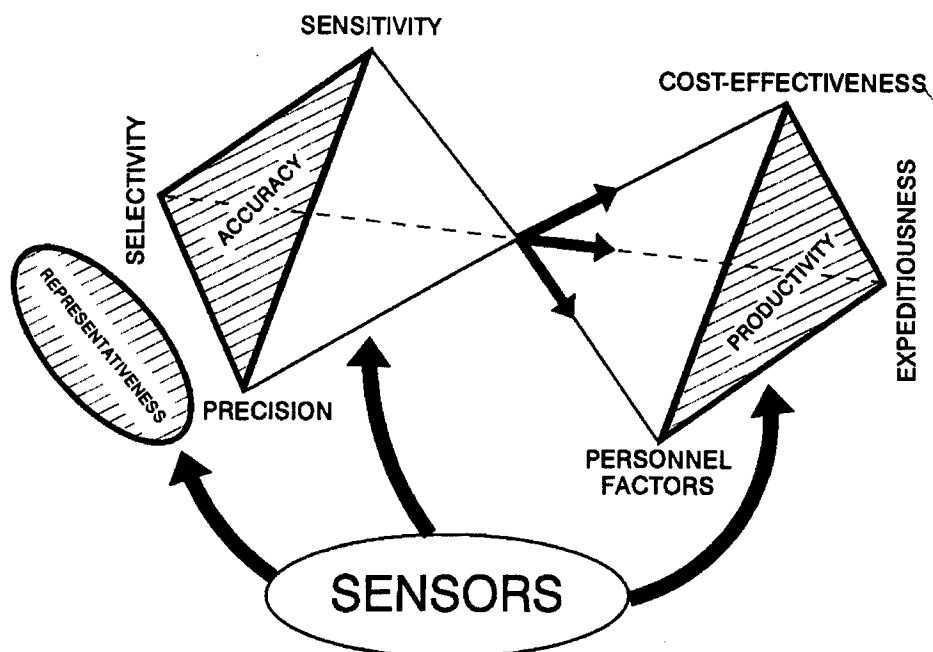
As noted earlier, on-line process monitoring is among the application areas that can benefit most from the use of sensors provided they can be incorporated into portable systems. Electrochemical sensors are generally more suitable for these applications, even though optical sensors can also be readily adapted for this purpose (*e.g.* by using LEDs or photodetectors instead of conventional photometers) at the expense of somewhat degraded spectral resolution and sensitivity.

Finally, for a (bio)chemical sensor to effectively solve real problems it should require no immediate interpretation of its response (*e.g.* in order to alter some physical or physico-chemical parameter influencing its operation). In practice, this requires that the sensor be reliably used by unskilled personnel, who often work under stressing conditions, in order to avoid the human factor as a source of error in the results produced by (bio)chemical sensors.

1.7 (BIO)CHEMICAL SENSORS AND ANALYTICAL PROPERTIES

For analytical properties to be consistent with the quality expected from an analytical process and the results derived from it, they should be considered in a hierarchical way [4,50]. Thus, there are three primary types of analytical properties, namely: (*a*) *capital properties* (accuracy and representativeness), which are directly related to quality of the results; (*b*) *basic properties* (sensitivity, selectivity and precision), which support accuracy and are related to the analytical process; and (*c*) *accessory properties* (expeditiousness, cost-effectiveness and personnel safety/comfort), which are also related to the properties are related to one another in an additive or contradictory way. The best way of envisaging the ensuing relationships is by means of two analytical tetrahedra sharing a common apex (see Fig. 1.16.A). The apices of the tetrahedron on the left hold the basic analytical properties that define the accuracy triangle, whereas those of the tetrahedron on the right accommodate the accessory analytical properties, which delimit the analytical

A)



B)

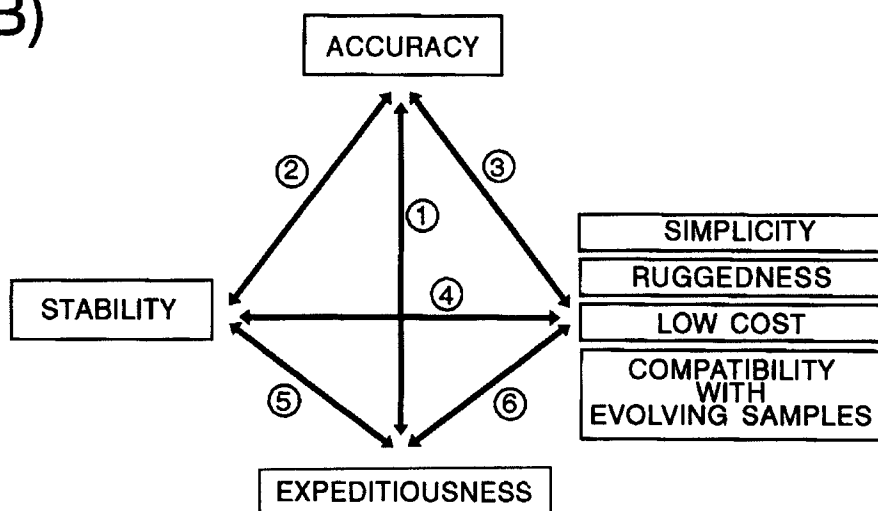


Figure 1.16 — (A) Impact of (bio)chemical sensors on analytical properties. (B) Analytical tetrahedron for (bio)chemical sensors.

productivity triangle. The capital property representativeness must be considered in isolation. These tetrahedra can also be used to demonstrate the contradictory relationships between properties. In fact, the symmetric shapes in Fig. 1.16.A are representative of an ideal, balanced situation; however, if special emphasis is to be placed on one, two or three properties, then it has to be to the detriment of the rest and the tetrahedra will be distorted as a result [50]. analytical process and define the so-called "analytical productivity". All these

(Bio)chemical sensors have an unlike impact on the three groups of analytical properties. As shown in Fig. 1.16.A, they are most influential on representativeness and the accessory analytical properties. In fact, if a sensor is brought into contact with an untreated, unmeasured, uncollected sample, the typical errors arising from sampling will obviously be minimized as a result; however, representativeness also entails consistency of the results with the analytical (and hence economic and/or social) problem addressed, which can hardly be influenced by the use of a sensor [51]. This is also the case with the tetrahedron for the basic analytical properties; sensors as such rarely increase accuracy (and hence sensitivity, selectivity or precision) relative to other modern analytical alternatives, even though they occasionally result in somewhat improved sensitivity, selectivity or precision with respect to a conventional analytical method based on the same reaction or separation (*e.g.* [52,53]). The greatest effect of sensors is exerted on analytical productivity; as noted in previous sections, sensors can efficiently contribute to increased expeditiousness, decreased costs and greater personnel safety and comfort. As a result, the symmetric picture shown in Fig. 1.16 is distorted and the tetrahedron on the right expands at the expense of that on the left.

It is also interesting here to present in graphical form the most salient properties of sensors in order to obtain a global view and facilitate establishment of mutual relationships. Figure 1.16.B shows the analytical tetrahedron for (bio)chemical sensors, which is adapted from the general hierarchical ordering [50]. The tetrahedron includes the most significant properties, but could equally have contained others specific to some types of (bio)sensors. Placement of accuracy, rapidity, stability and "other properties" at the four apices is intended to show their opposing relationships; if emphasis in a given analytical process to be implemented by use of a (bio)chemical sensor is placed on one, two or three of the properties, it has to be at the expense of the remainder, so one of the tetrahedron apices, edges or sides will be distorted as a result. Enhancing the capital property

accuracy will usually involve decreasing the area of the stability–expeditiousness–others triangle, even though it may also affect stability and robustness. Increased response expeditiousness can be achieved at the expense of diminished accuracy, stability and accessory characteristics. The experimental conditions under which the sensor is stable may not coincide with the optimal conditions leading to maximal levels of the remaining properties. This is also the case if emphasis is placed on robustness or analytical cost-effectiveness (the operational conditions would be different from those resulting in optimal levels of the other properties). These and other examples show the mutual relationships between sensor properties, which no doubt determine planning and execution of the associated analytical process.

1.8. COMMERCIAL AVAILABILITY

Commercial availability is an unquestionable indicator of the utility and potential of any analytical chemical technology and follows a more or less prolonged period of basic developments. Despite their doubtless impact and potential quality, as well as the large number of academic achievements reported, relatively few (bio)chemical sensors are still commercially available. The basic reason for this scarcity is very simple: available (bio)chemical sensors provide too low levels of the properties dealt with in Section 1.6 for massive, reliable, effective use in routine applications intended to solve real analytical problems. In addition, dedicated researchers have preferentially focussed endeavours on academic developments and ignored potential practical uses. It is interesting to note that few leading analytical instrumentation manufacturers include any (bio)chemical sensors in their ranges. Biosensors, which are subject to more marked stability problems, are becoming increasingly frequently available in commercial form, particularly for clinical applications in response to the steadily growing demands from the clinical area. The recently published review by Alvarez–Icaza *et al.* [54] is of special interest in this context. Table 1.1 lists selected commercially available biosensors, some of which are not sensors proper, but sensor systems (*e.g.* FI assemblies including an enzyme reactor and an ordinary flow-cell for electrochemical measurements by means of an integrated electrode, or photometric measurements). Note the prevalence of electrochemical transducing, which also plays a prominent role in chemical sensors; in fact, there is now a sizeable market for ion-selective electrodes

Table 1.1. Selected commercially available biosensor systems^(*)

Company	Model	Analyte	Principle	Application
Genetics International, U.K.	Exac Tech	Glucose	Disposable mediated enzyme electrode	Blood
Prügerätewerk Mendigen GmbH, Freital, Germany	ESAT 6660-2	Glucose, lactate	Enzyme electrode	Blood, serum, plasma
Metertech Inc. Nan Kang Taipei, Taiwan	Model 5000	Glucose	Glucose strip	Blood
Yellow Springs Instrument Co., USA	2700 Select	Glucose, lactate, ethanol, lactose, sucrose, galactose, methanol, starch	Enzyme electrode	Biotechnology, pharmaceuticals, foods
	2300 Stat	Glucose, lactate	Enzyme electrode	Blood, plasma, serum
	1500 G	Glucose	Enzyme electrode	Blood, plasma
	FGA-1 Glucose Analyzer	Glucose	Enzyme electrode + flow injection analysis	Biotechnology
TOA Electronics Ltd., Tokyo, Japan	Glu-11	Glucose	Enzyme electrode	Food, medicine

Kalger GmbH, Neuberg, Germany	Microzym-L	Lactate	Enzyme electrode	Food, biotechnology, medicine
Electrolux Fermentation Getinge AB, Getinge, Sweden	Electrolux	Glucose	Enzyme reactor + flow injection analysis	Biotechnology
Sigma, Russia	EXAN	Glucose	Enzyme electrode	
Dosivit, Nantes, France	MC2 Multisensor	Glucose, sucrose, lactose, lactate, ethanol	Electrochemical enzyme sensor	Agriculture, food
La Roche, Switzerland	LA 640	Lactate	Enzyme electrode	
Aucoteam GmbH, Berlin, Germany	BODyPoint	BOD	Microbial electrode	Waste water
Central Kagaku Corp., Tokyo, Japan	BOD-2000	BOD	Microbial electrode	Sewage and waste water
Kelma, Niel, Belgium	RODToX	Short time BOD, toxicity	Microbial reactor + O ₂ electrode	Municipal and industrial waste water
Pharmacia Biosensor AB, Uppsala, Sweden	BIAcore		Immunologic surface plasmon resonance	Biomolecular interactions

(*) Reproduced from [54] with permission of the American Chemical Society

(ISEs) whereas ion-sensitive field-effect transistors (ISFETs) are just starting to be launched. On the other hand, few optical chemical sensors have so far been commercialized, even though some prominent manufacturers already market fibre-optic modules for fitting to various molecular spectroscopic instruments, which can be regarded as the first step to a massive development of this type of sensor. Very few mass and thermal sensors have crossed the threshold between academic developments and routine applications. This situation is consistent with the stage of basic development reached by the different types of sensors arranged according to type of transducing element shown in Fig. 1.11.

1.9 TRENDS IN SENSOR DEVELOPMENT

In appraising trends in some scientific or technical area, one usually tends to confuse the real and the ideal, *i.e.* what is being or will foreseeably be achieved and what is actually needed. This conflict also reaches what is discussed in this section, where the two sides are frequently mixed. There follows a description of major general trends in (bio)chemical sensors with explicit exclusion of those involving a specific group of sensor.

First of all, the already intensive research into sensors should be re-orientated in order to adapt what has already or is currently being developed to facts. (Bio)chemical sensors should be endowed with practical use, which somehow entails replacing brilliant academic achievements with a more pragmatical approach. This trend could be fostered by the editors of scientific journals, who should reject publication of scarcely innovative material (*i.e.* slight variations on an existing theme) and encourage submission of papers clearly showing the use of sensors for solving real analytical problems. Also, the R&D departments of large scientific instrument manufacturing firms should start to consider (bio)chemical sensors as a serious alternative to conventional instruments. In fact, many of the currently available (bio)chemical sensors are marketed by small or medium-size firms.

As regards general technical features, (bio)chemical sensors will foreseeably result in increased automation, simplification and miniaturization, three of their inherent attributes (Fig. 1.8). Operationally, those features assuring quality in the results (Fig. 1.16.B), as well as others including durability, expeditiousness, robustness, cost-effectiveness, compatibility with complex samples, and ease of incorporation into portable analytical systems

are in need of improvement. Fulfillment of these objectives would no doubt accelerate massive commercialization.

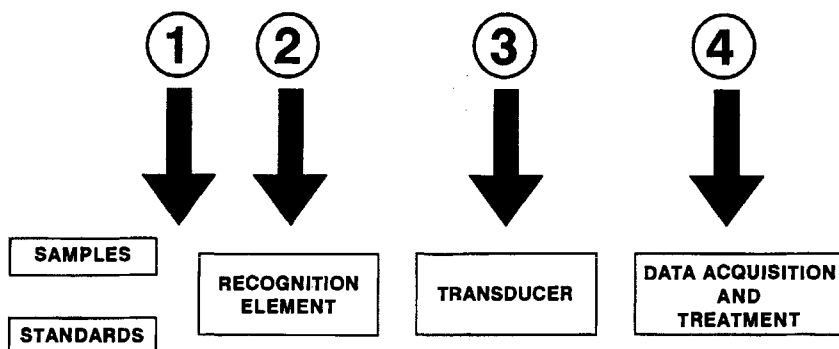


Figure 1.17 — Focal points for research and development in (bio)chemical sensors. For details, see text.

Figure 1.17 shows a schematic diagram of the preferential focal points for basic and applied research into (bio)chemical sensors, which are briefly discussed below.

The sample/sensing microzone interface (Fig. 1.17.1) has so far rarely been considered in a systematic manner notwithstanding its great practical significance. As noted earlier, few samples (or standards) can be brought into direct contact with a (bio)chemical sensor, so some pretreatment (*e.g.* macromolecule separation, dilution, pH adjustment, addition of a conditioner) is almost invariably required. In fact, one of the most salient assets of flow-through sensors is their (still scarcely explored) potential for on-line coupling to continuous-flow configurations. The advantages arising from such coupling are shown graphically in Fig. 2.11. This is also the case with calibration, for which probe-type sensors require a robot arm if human intervention is to be avoided; on the other hand, a flow-through sensor coupled on-line to a continuous configuration allows automatic, convenient, effective calibration. This step of the analytical process is particularly critical with probe-type (bio)chemical sensors used for in-line monitoring, which are cumbersome to withdraw from the monitored evolving sample and call for

different experimental conditions than those used to monitor the analyte in the sample.

Many authors engaged in (bio)chemical sensor research have focussed their interest in the sensing microzone (Fig. 1.17.2), which is the ultimate origin for such major properties as sensitivity, selectivity, precision, expeditiousness and robustness. Biosensors have a predictably promising future in relation to protein engineering, catalytic antibodies, chemoreceptors and supramolecular chemistry. Research into chemical sensors is essentially being aimed at the search for selective recognition processes since the traditional use (and abuse) of non-specific systems has little practical future. The recognition elements of both types of sensor have been the target of miniaturization attempts (*e.g.* by use of thick-film and thin-film technologies) with the purpose of reducing sensor size (*e.g.* for *in vivo* monitoring applications) or improving its performance. Attempts at minimizing the influence of transport phenomena (*e.g.* diffusion) and, in general, shortening the response and regeneration time, are also noteworthy. On the other hand, basic research aimed at characterizing the sensing element and studying its changes by use of sophisticated analytical techniques has a promising future in this context [55].

As stated above, miniaturization of the transducing system (Fig. 1.17.3) is also important as it allows sensors to be incorporated into portable systems for field applications. However, this has so far resulted in diminished performance from "shrunk" transducers, which should be avoided in the future. One other trend as regards transducers is a gradually increasing use of optical (mostly photometric and fluorimetric) systems, as can be seen from Fig. 1.11. The advantages and disadvantages of optical (bio)chemical sensors have been the subject of much controversy as they are obviously appraised differently by authors depending on whether or not they are active workers in the field. Most comparisons in this respect involved electro-analytical (bio)chemical sensors, the most serious contenders for optical sensors in terms of consolidation. These assets and pitfalls were compared in a recent review [37]. It is worth emphasizing here the work of the research group formerly headed by the late Professor Simon, who enjoyed wide acknowledgement among electrochemical sensor (ISE) specialists and devoted his last energy to developing flow-through optical sensors to which he contributed his expertise in chemical response mechanisms of the recognition element. A detailed description of such sensors is provided in Chapter 5.

No doubt, computer science in general and chemometrics in particular offer a vast potential for Analytical Chemistry to dramatically raise the amount and quality of information that can be abstracted from raw data. The use of smart signal-processing systems is one of the more salient trends in the context of (bio)chemical sensors (Fig. 1.17.4).

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2

Fundamentals of continuous-flow (bio)chemical sensors

2.1 DEFINITION

The most salient feature of flow-through sensors is the way in which the sample is brought into contact with the sensitive microzone (see Fig. 1.14), which distinguishes them from probe and drop-planar sensors. In fact, the liquid (or gaseous) sample is passed over the microzone rather than dropped onto it or used to immerse the probe [1].

In broad terms, a flow-through sensor is an analytical device consisting of an active microzone where one or more chemical or biochemical reactions, in addition to a separation process, can take place. The microzone is connected to or incorporated into an optical, electric, thermal or mass transducer and must respond in a direct, reversible, continuous, expeditious and accurate manner to changes in the concentrations of chemical or biochemical species in the liquid or gaseous sample that is passed over it, whether forcefully (by aspiration or injection) or otherwise (gases).

At this point it is worth setting a clear distinction between continuous-flow analytical systems (occasionally referred to as "sensor systems") and flow-through sensors, two terms that are often confused in the analytical literature. The primary difference between them lies in whether or not detection is performed simultaneously with other steps in the continuous system. Figures 2.1 and 2.2 illustrate the differential features of the two types of system.

In conventional continuous-flow configurations [2,3], (bio)chemical reactions, separations based on mass transfer between two phases, and continuous detection occur at different places and hence sequentially.

(Bio)chemical reactions may take place prior to or after the continuous separation module and are intended to enhance or facilitate mass transfer, detection or both. The earliest and simplest approach to integrated analytical steps in continuous-flow systems involves a combination of chemical reactions and continuous separations [4,5]. Such is the case with the formation of soluble organic chelates of metal ions in liquid-liquid extractions with the ligand initially dissolved in the organic stream [6], the formation and dissolution of precipitates [7], the formation of volatile reaction products in gas diffusion [8] and that of volatile hydrides in atomic absorption spectro-

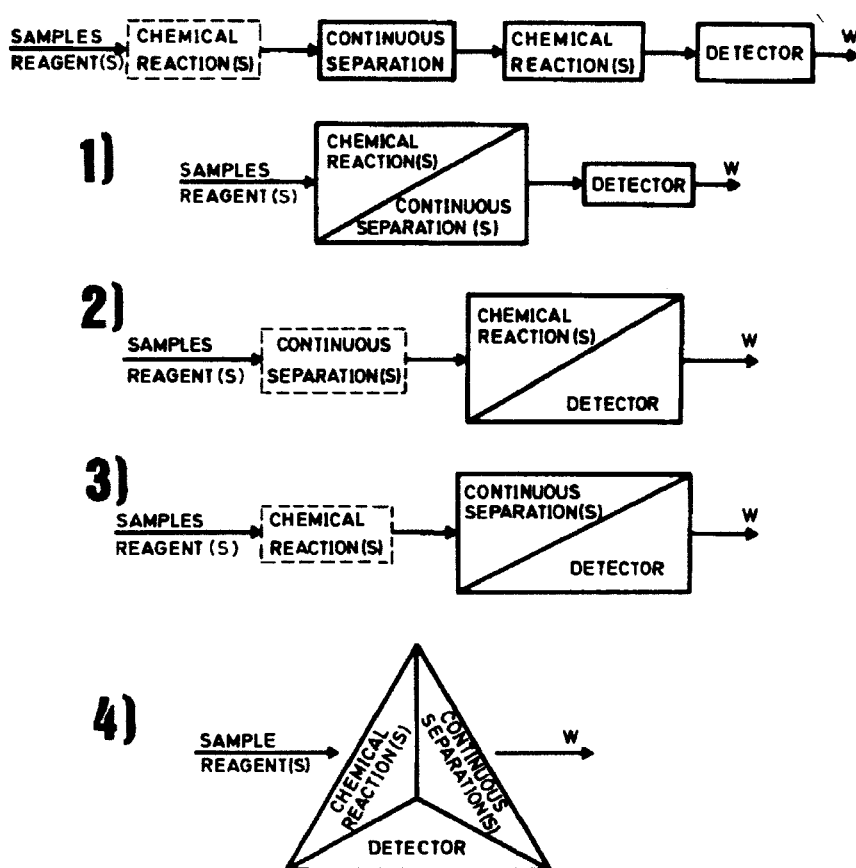


Figure 2.1 — Variants of integrated reaction, separation and detection in continuous-flow analytical systems. (1) Reaction/separation. (2) Reaction/detection. (3) Separation/detection. (4) Reaction/separation/detection.

scopy [9]. However, continuous systems with integrated reaction and separation cannot be considered sensors since detection is not implicitly integrated and takes place in a flow-cell (or in the detector itself in atomic spectroscopic techniques) at the end of the continuous system.

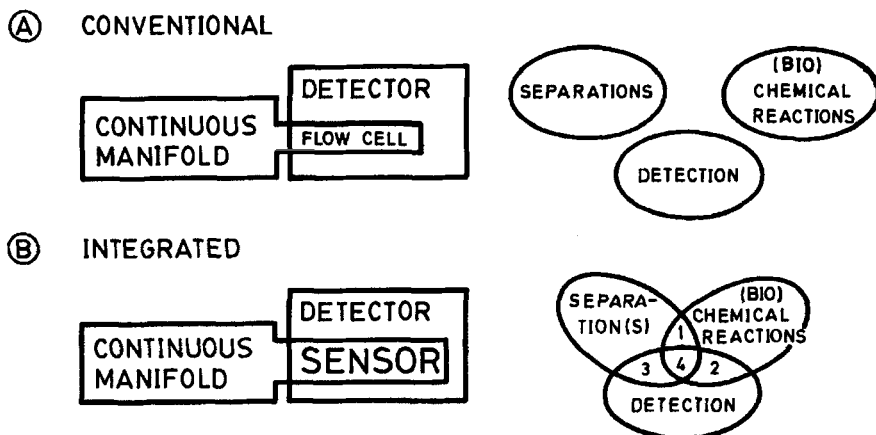


Figure 2.2 — Differences between ordinary continuous systems (A) and those involving integration of detection with some other step of the analytical process (B). Numbers 1–4 match those in Fig. 2.1. (Reproduced from [1] with permission of the Royal Society of Chemistry).

Those systems where detection is integrated with other analytical steps (chemical reactions, separations or both) can indeed be regarded as (bio) chemical sensors since they conform to the definition given in Chapter 1. Such systems may involve previous, non-integrated continuous separations and (bio)chemical reactions implemented in individual modules. Thus, detection in a continuous-flow (bio)chemical sensor takes place simultaneously with reaction (type 2), separation (type 3) or both (type 4) —see Figs 2.1 and 2.2. This definition excludes some obvious features of sensors such as miniaturizability, portability and compatibility with straightforward detectors. Thus, some continuous integrated separation–detection systems [10] that conform to the definition of sensor are not strictly true sensors in practice. Such is the case with the stripping electrochemical devices recently reviewed by Luque de Castro and Izquierdo [11], which integrate both steps (even though they occur sequentially), some integrated gas-diffusion/atomic absorption spectroscopy [12] and liquid–liquid extraction/photometry systems [13]. In other words, the fact that a given continuous system integrates

detection and one or more steps of the analytical process does not warrant calling it —or some part of it— a sensor.

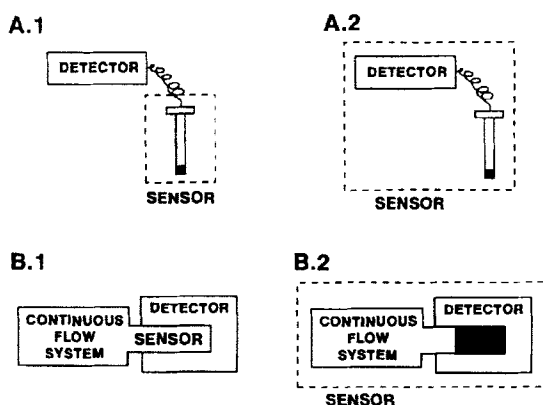


Figure 2.3 — Different uses of the word "sensor" (1,2) to refer to probe (A) and flow-through sensors (B) according to various criteria.

What is the actual meaning of the word "sensor" then? In probe sensors (Fig. 2.3.A) the word is used to refer to both the probe, accommodating the sensitive microzone, and the whole device (transducer included). This is also the case with flow-through sensors (Fig. 2.3.B), where "sensor" denotes either the flow-cell that contains the sensitive microzone or the entire continuous-flow system. Which usage is the more correct? On the one hand, it seems logical to use the word sensor to designate the probe or flow-cell including the active microzone (Fig. 2.3.A) as this marks a distinction from batch and continuous conventional analytical systems. On the other, neither a probe nor an active flow-cell can deliver any analytical information, condition the sample or regenerate the microzone without the concurrence of the detector and other system components.

2.2 CLASSIFICATION

The classifications of sensors established in Chapter 1 can be used as guidelines to define various technical categories of flow-through sensors (Fig. 2.4).

One possible classification is based on the type of physico-chemical phenomena that may occur in the sensor. Based on this criterion, there are passive flow-through sensors, which possess no reactive microzone and are

used for direct measurements based on intrinsic physico-chemical properties of the analytes. They are not substantially different from the most ordinary probe sensors, whether optical (*e.g.* those based on absorbance and nascent fluorescence measurements) or electrochemical (potentiometric, voltammetric), except in the way they are brought into contact with the sample. Figure 2.5 compares the different types of passive sensors; as can be seen, flow-through sensors of this type are not essentially different from ordinary continuous-flow configurations (*e.g.* flow injection manifolds) either.

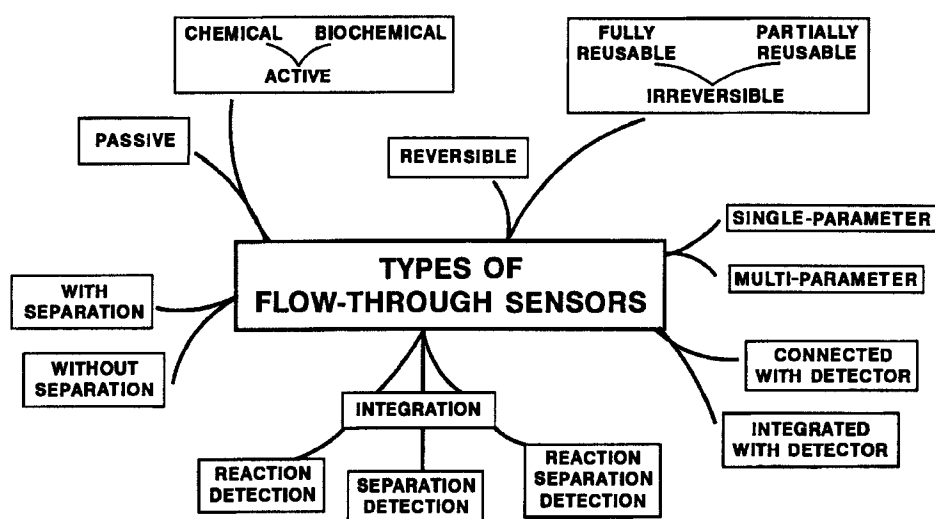


Figure 2.4 — Classifications of flow-through (bio)chemical sensors.

Most flow-through (bio)chemical sensors rely on chemical and physico-chemical interactions between the analyte and an active microzone that induce changes to which the sensor is responsive. Such interactions may be chemical or biochemical depending on the nature of the reaction ingredients, the analyte and the species immobilized at the microzone. Mass transfer between two phases, with or without a concurrent chemical reaction, is also possible in this situation. Therefore, based on the type of process that takes place at the time of sensing, flow-through sensors can be classified into three broad categories according to whether detection is integrated with a (bio)chemical reaction, a separation or both. This classification is adopted throughout the book in dealing with the different types of sensors.

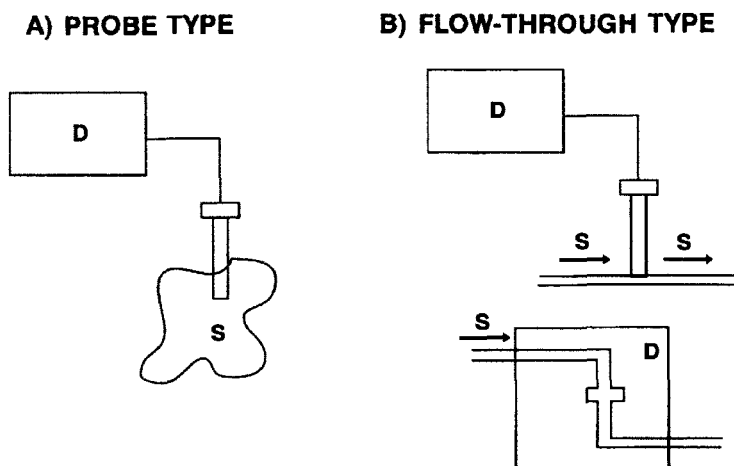


Figure 2.5 — Probe (A) and flow-through (B) passive sensors. S: sample. D: detector.

Not many flow-through sensors meet reversibility requirements, *i.e.* the signals they produce do not always change continuously in response to variations (increases or decreases) in the analyte concentrations. Such is the case with potentiometric electrodes used in flow-cells (*e.g.* see [14]) and sensors including an immobilized catalyst, where the biochemical reaction involved takes place very rapidly (*e.g.* see [15]). Many of the flow-through sensors reported so far are irreversible and reusable, and operate in two steps (measurement and regeneration) that can be readily and conveniently implemented thanks to the continuous operational mode use. On the other hand, there are very few irreversible non-reusable flow-through (bio)chemical sensors, of which those using an immobilized reagent that is consumed during sensing —the sensor can thus only be used a few times— are somewhat more common (*e.g.* see [16]).

One other, very descriptive classification of flow-through sensors is based on the location of the active microzone and its relationship to the detector. Thus, the microzone can be connected (Figs 2.6.A and 2.6.B) or integrated (Fig. 2.6.C) with the measuring instrument. Sensors of the former type use optical or electric connections and are in fact probe sensors incorporated into flow-cells of continuous analytical systems; they can be of two types depending on whether the active microzone is located at the probe end (*e.g.* see [17]) or is built into the flow-cell (*e.g.* see [18]) —in this latter case,

the sensor can be considered a passive probe for monitoring changes in the active microzone on passage of the sample. In integrated flow-through sensors, the active microzone is located in the flow-cell of a conventional non-destructive detector (a photometer or fluorimeter), so they are scarcely different from ordinary continuous-flow systems (*e.g.* see [19]).

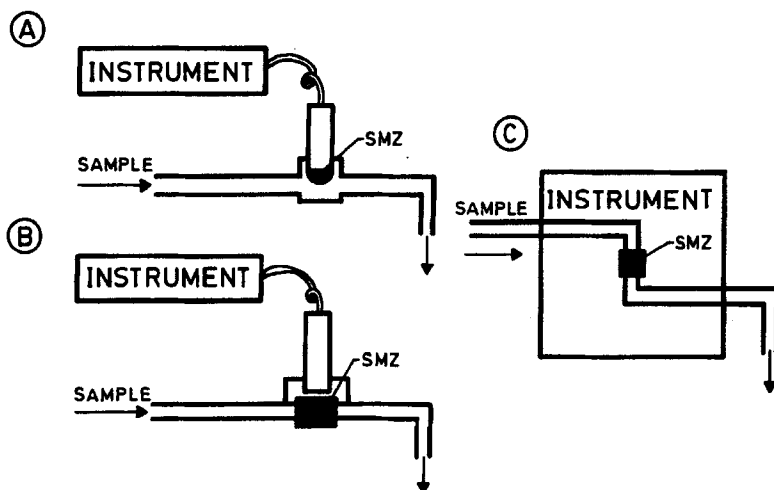


Figure 2.6 — Classification of flow-through sensors according to the location of the active microzone relative to the measuring instrument: (A,B) connected; (C) built-in. (Reproduced from [1] with permission of the Royal Society of Chemistry).

Depending on their sensing ability, flow-through sensors can be classified into single-parameter and multi-parameter according to whether they can sense one or more analytes on passage of each sample. Multideterminations usually rely on the use of an instrument (*e.g.* a diode array spectrophotometer) for virtually simultaneous multidetection (*e.g.* [20]).

Other possible classifications of flow-through sensors have been excluded from Fig. 2.4 because they are either of little consequence or dealt with in other sections below. Such is the case with the classification based on whether one or more of the active reaction ingredients (analyte, reagent, catalyst, reaction product) is immobilized temporarily or permanently on the active microzone. In addition, the immobilization process may involve one or several active components.

2.3 THE ACTIVE MICROZONE

Active flow-through (bio)chemical sensors include a microzone where a (bio)chemical reaction, a separation or both takes place. The active microzone may be located in the flow-cell itself (Figs 2.6.B and 2.6.C) or built into a probe sensor for insertion into a continuous-flow analytical system (Fig. 2.6.A). The external appearance of a sensitive microzone can be as widely different as the type of detector and process concerned. This is discussed in greater detail in the following section.

The way in which the active microzone is retained also depends on its relationship to the detector (Fig. 2.6) and the type of interaction with the analyte or its reaction product. If the microzone is an integral part of the probe, an additional support (usually a membrane) is often required, so contact with the sample is hindered to some extent. On the other hand, a microzone located in a flow-cell can be retained in various ways. Thus, if the microzone consists of a porous solid or particle, the flow-cell is simply packed with two filters in order to avoid washing out (*e.g.* see [21]). Too finely divided solids (*viz.* particle sizes below 30–40 μm) should be avoided as they require pressures above atmospheric level, which complicates system design and precludes use of microzones with a high specific surface. Placing a separation membrane in a flow-cell is

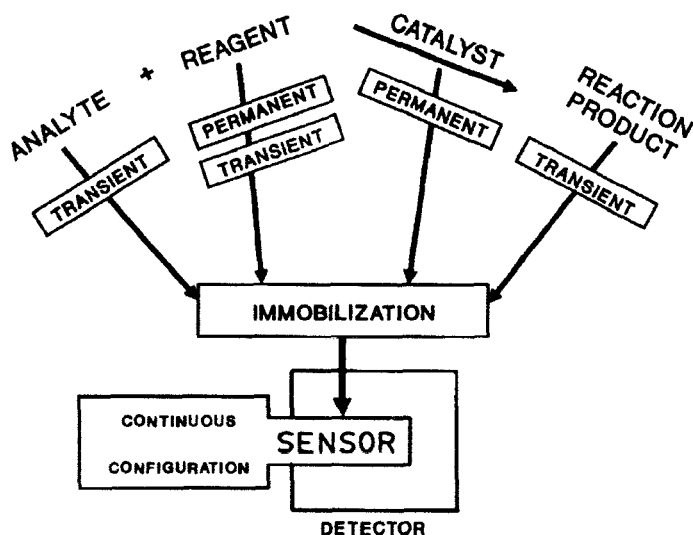


Figure 2.7 — Types of species retained and immobilization at the active microzone of a flow-through sensor.

fairly simple if the latter consists of two detachable parts: the membrane need only be positioned between both parts in a "sandwich" arrangement (*e.g.* see [22]). Occasionally, membranes are prepared *in situ* by evaporating a polymer solution onto the flow-cell.

The "activity" of a microzone in a flow-through sensor involving separation alone can be generated by immobilizing the analyte or its reaction product temporarily on an appropriate support. Some such sensors (see Chapter 4) involve no immobilization, but mass transfer across a membrane [10]. However, the microzone activity normally arises from immobilization of one or more of the (bio)chemical reaction ingredients (the analyte, reagent, catalyst or reaction product), as shown in Fig. 2.7. The ingredient(s) in question can be immobilized temporarily (the species retained is partly or completely removed) or permanently. When the immobilized species is the analyte or its reaction product, immobilization is temporary or transient, which makes flow-through sensors operating in two steps (retention/elution) integrated with detection irreversible and reusable. Temporary immobilization is also customary for reagents that are consumed in the process; under such conditions, the sensor can only be used a limited number of times (*e.g.* see [16]).

When the active species is to be reused many times, they must be immobilized permanently at the active microzone, which is how the reagent and catalyst are usually immobilized. An immobilized reagent must act in a reversible manner or be regenerable (usually by analyte removal or elution); on the other hand, a catalyst is self-regenerating, so no external action is required to make the sensor reversible.

It should be noted that immobilization on the active microzone can occasionally be both permanent and temporary; such is the case when two reagents (*e.g.* see [23]) or a catalyst plus the reaction product (*e.g.* see [24]) are to be immobilized. Double immobilization is also common practice when the immobilized reagent retains the analyte and gives rise to a detectable alteration (a colour, fluorescence, mass or heat energy change) of the sensitive microzone (*e.g.* see [19]); all three processes (reaction, separation and detection) take place simultaneously rather than sequentially (see Chapter 5).

(Bio)chemical reactions can take place in various parts of flow-through sensors depending on the type of immobilization involved, namely:

- (a) In the active support when the analyte or reagent is immobilized (in a transient or permanent manner, respectively). In the former case, the reaction may involve an immobilized reagent (*e.g.* see [25]) or one passed at a later stage over the support onto which the analyte is retained (*e.g.* see [26]).
- (b) In the solution held in the flow-cell when the sensing microzone accommodates an immobilized catalyst (*e.g.* see [27]) or the cell holds an acceptor solution of the reagent which is separated from the carrier solution containing the sample by means of a membrane (*e.g.* see [28]).
- (c) In a previous zone (*e.g.* a "reactor" in the flow system) when the reaction product is temporarily immobilized (*e.g.* see [29]).

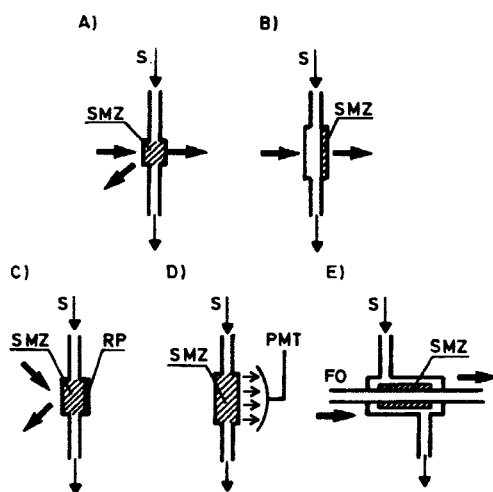


Figure 2.8 — Standard types of optical flow-through sensors. S. sample. SMZ: sensitive microzone. FO: fibre optics. For details, see text. (Reproduced from [1] with permission of the Royal Society of Chemistry).

2.4 FLOW-THROUGH CELLS

Depending on the type of detection involved and the process taking place at the active microzone (reaction and/or separation), the flow-cell that contains the microzone can exist in a variety of configurations [1], all

of which are integrated with or connected to a measuring instrument in such a way that at least one of the above-mentioned processes takes place simultaneously with detection.

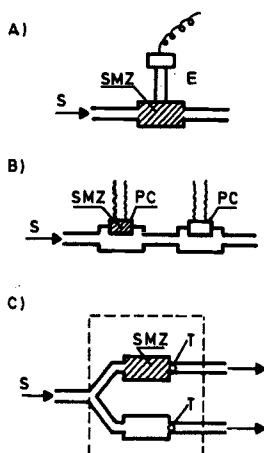


Figure 2.9 — General types of continuous-flow electric, thermal and mass sensors. S: sample. SMZ: sensitive microzone. E: electrode. PC: piezoelectric crystal. T: thermistor. For details, see text. (Reproduced from [1] with permission of the Royal Society of Chemistry).

Figure 2.8 depicts some of the more common configurations of optical flow-through sensors integrated with or connected (via fibre optics) to a detector. Molecular absorption measurements can be made in configurations A and B, which differ in the location of the active microzone (*e.g.* see [19,30]). On the other hand, molecular fluorescence measurements can only be made in configuration A (*e.g.* see [9]). Configuration C, where one of the cell sides includes a reflection plate, is frequently used for reflectance measurements. The simplest configuration, D, is used for (bio)chemiluminescence measurements, which only require the sensitive microzone to be placed opposite the photomultiplier tube (*e.g.* see [16]). Some fibre optic waveguides coated with a sensitive microzone (E) and placed in the flow-cell through which the sample is circulated were recently reported (*e.g.* see [32]).

The most frequently used electrochemical flow-through sensors are depicted schematically in Fig. 2.9.A; the electrode is brought into contact with the sensitive microzone, which is accommodated in the flow-cell

(*e.g.* see [33]) or attached to the surface of the working electrode (*e.g.* see [34]). The reference and counter electrode can be placed in the cell or a different place in the continuous assembly.

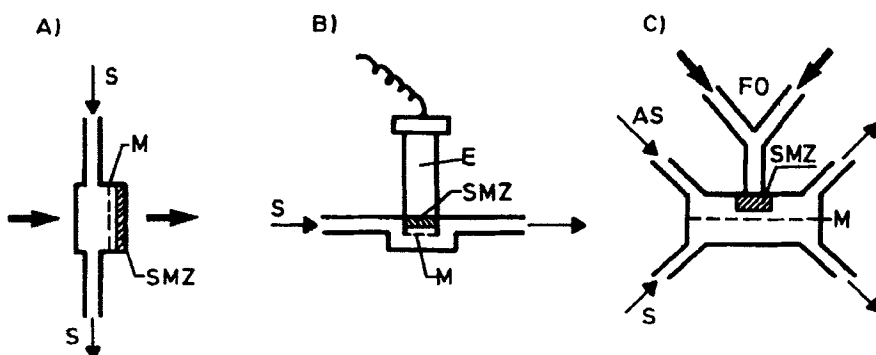


Figure 2.10 — Standard types of optical flow-through sensors. S: sample. SMZ: sensitive microzone. FO: fibre optics. For details, see text. (Reproduced from [1] with permission of the Royal Society of Chemistry).

Thermal and mass flow-through sensors rely on differential measurements owing to the low selectivity of these types of detection. They use two flow-cells arranged in series (Fig. 2.9.B) or parallel (Fig. 2.9.C), each containing a sensitive microelement (a piezoelectric crystal or a thermistor). One of the cells houses the sensitive microzone, whereas the other is empty or accommodates an inert support containing no immobilized reagent (*e.g.* see [35]).

Membrane flow-through sensors (Fig. 2.10) are worth special note as their configuration must be adapted to the separation process concerned and the type of detector used. There are two main types differing in whether the flow-cell is crossed by one or two streams. In the simplest possible configuration, only the sample stream is passed through the cell, isolated from the sensitive microzone by the membrane; detection can be optical (A) (*e.g.* see [36]) or electroanalytical (*e.g.* see [37]). Alternatively, a sandwich configuration (C) can be used with the membrane isolating the donor (sample) stream from the acceptor stream, where detection is to take place (*e.g.* see [22,28]). Provided the kinetics of mass transfer is fast enough, the sensor membrane requires no regeneration, unlike those involving retention of the analyte at an active surface.

2.5 CONTINUOUS CONFIGURATIONS

One of the most valuable assets of flow-through (bio)chemical sensors is their compatibility with unsegmented-flow configurations, which endows them with major advantages over probe-type sensors including higher flexibility and automatability in addition to wider applicability to real rather than academic problems —the former are rarely addressed by using sensors.

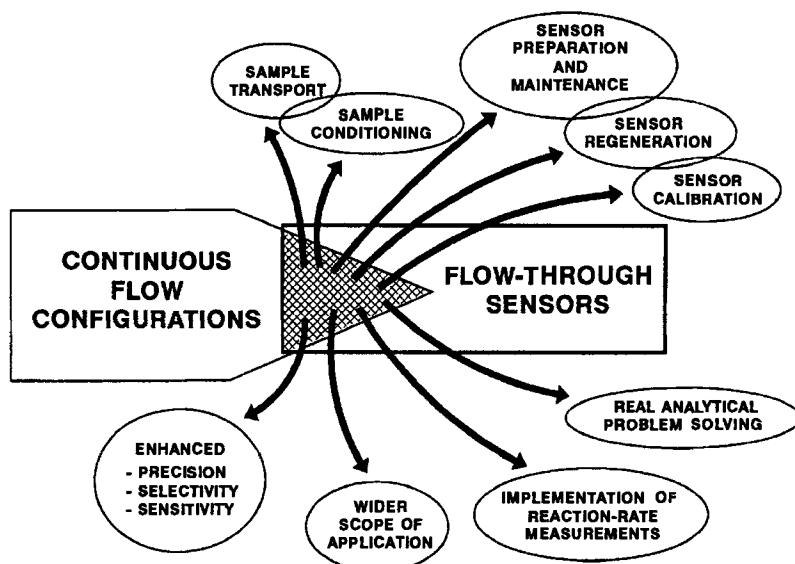


Figure 2.11 — Functions and implicit advantages over conventional probe sensors of continuous configurations coupled on-line to a flow-through (bio)chemical sensor.

A continuous configuration used in conjunction with a sensor can serve a variety of functions depending on the nature of the sample and analyte, the type of sensor concerned (*viz.* on the active microzone and immobilized species) and the type of integrated or connected detector. Figure 2.11 summarizes such functions, namely: (A) transferring the injected or aspirated sample to the sensor; (B) conditioning the sample (pH adjustment, mixing with other reagents, masking) for optimal development of the process (reaction and/or detection) that is to take place at the active microzone; (C) maintaining or preparing the sensor

for use; (D) regenerating the sensor between samples (or samples and standards) if it is of the regenerable-reusable type; (E) facilitating straightforward, reliable calibration; (F) increasing the sensor selectivity and sensitivity via a continuous separation module; (G) boosting precision through reduced human participation in sensor-related operations; (H) extending applicability to other analytes giving rise to the same reaction products for which the sensor was originally designed; (I) allowing the development of kinetic methodologies based on differential rather than absolute measurements; and (J) filling the gap between sensor development and real analytical problems.

Various unsegmented continuous configurations coupled on-line to flow-through sensors are discussed below. They are dealt with in groups according to whether immobilization is transient or permanent, which determines the type of process involved. A distinction is made between reversible and irreversible-reusable sensors, but no mention is made of those sensors in which some immobilized reagent is lost during sensing.

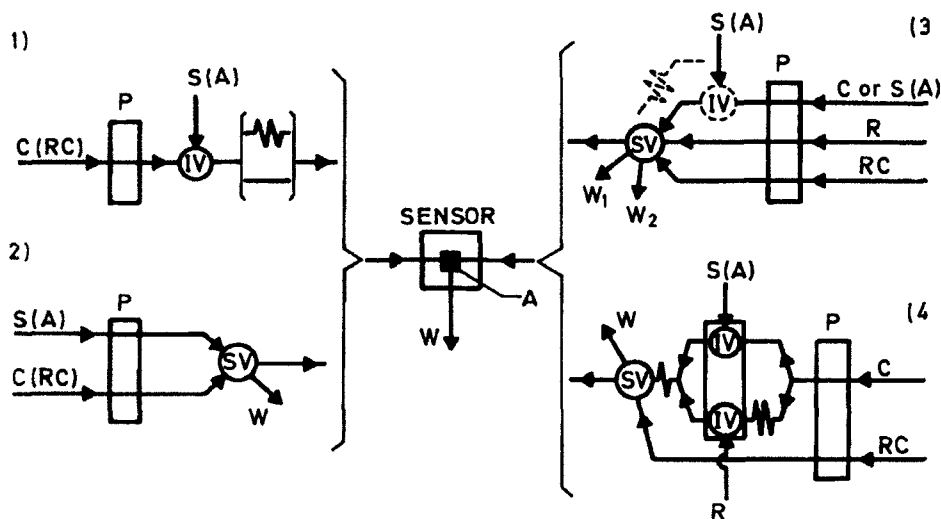


Figure 2.12 — Continuous configurations coupled on-line to flow-through sensors involving transient immobilization of the analyte (A) contained in the sample (S). P: pump. C: carrier. RC: regenerating carrier. R: reagent. IV: injection valve. SV: switching valve. W: waste. For details, see text.

Figure 2.12 shows some of the more commonly used configurations with *sensors based on transient immobilization of the analyte* at the active microzone. Two main types are distinguished according to whether the physico-chemical properties of the analyte are used to obtain the signal (types 1 and 2) or a reagent is employed to start a (bio)chemical reaction after the analyte is retained in order that the product may form *in situ* at the active microzone (types 3 and 4). The process may involve a single step (reversible sensors), two steps (irreversible-regenerable sensors) or three steps (the analyte is retained first and the reaction product is formed and then eluted). With reversible sensors, the sample can be injected (type 1) or continuously aspirated (type 2) —in the latter case, the carrier (C) can function to condition the sample with the aid of a mixing coil located after the injection valve or the sensitive microzone. With irreversible-reusable sensors, the carrier (RC) must act as the sensor regenerator, which can be accomplished in two ways, namely: (a) by using an unusually large injected volume for FIA (300–2000 μL) and transferring it virtually free from the carrier to the detector, which will respond to it and be regenerated as the sample ceases to pass through it; and (b) by using a switching valve for sequential introduction of sample and regenerating carrier (type 2). If the sensor is fully reversible and neither itself nor the sample require conditioning, then configuration 2 less the switching valve is typically used. The reagent required to reveal the analyte retained by the sensor can be introduced by using a switching valve to sequentially insert sample, reagent and regenerating carrier streams (type 3), even though the sample can also be injected into a conditioning carrier. If the reagent is scant or expensive, an asymmetric merging zones configuration (type 4), based on injection of two microvolumes of sample and reagent into two streams of the same carrier is favoured; the sample plug is passed before the reagent since the tube length connecting the reagent injection port to the merging point is longer. In this way, the analyte and reagent reach the sensor sequentially. The sensor is regenerated at a later stage similarly as in the above-described configurations.

Figure 2.13 shows the more commonly used on-line configurations with *flow-through sensors including a permanently immobilized reagent*. The analyte can interact with the immobilized reagent in two chief ways, namely: (a) by yielding a reaction product (*e.g.* a chelate), which requires the prior temporary immobilization of the analyte and subsequent elution

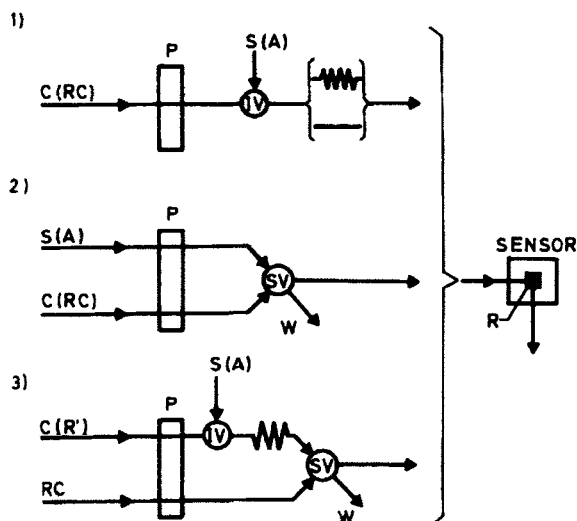


Figure 2.13 — Continuous configurations coupled on-line to flow-through biochemical sensors involving permanent immobilization of the reagent (R) at the active microzone. Symbol meanings are given in Fig. 2.12. For details, see text.

of the product and regeneration of the sensor; and (b) by inducing a physico-chemical change in the reagent, which will return to its original state as the plug or stream containing the analyte ceases to pass through the sensor. With reversible sensors, the sample can be injected (type 1) or aspirated (type 2) into the continuous configuration, where the carrier (C) will be used to transport the injected plug, condition the sample (type 1 configurations require an additional mixing coil for this purpose) or condition the reagent (type 2 configurations). On the other hand, the carrier used with an irreversible-reusable sensor contains a substance that regenerates the sensor, either in the sample introduction step in type 1 configurations (a large sample volume and a short connecting tube between the injection valve and the sensor are used) or in two steps in type 2 configurations (the sample and regenerating carrier streams are inserted with the aid of a switching valve). Type 3 configurations have some elements in common with the previous two. The sample can be conditioned on injection by mixing it with the carrier, which may contain an additional reagent for proper sensor performance. Also, the sensor is

regenerated at a second step by injecting a regenerating carrier solution with the aid of a switching valve.

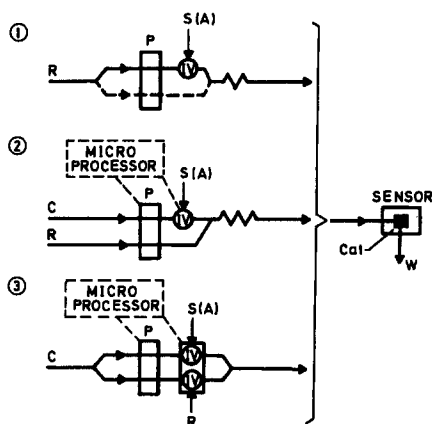


Figure 2.14 — Continuous configurations coupled on-line to flow-through (bio)chemical sensors involving permanent immobilization of the catalyst (Cat) at the active microzone. Symbol meanings are given in Fig. 2.12. For details, see text.

Figure 2.14 depicts the configurations more commonly used in on-line combinations with flow-through biochemical *sensors where the catalyst is immobilized in a permanent manner* on the active microzone. These configurations are primarily used for transferring the analyte sample–reagent suit under optimal conditions to the active microzone. The associated sensors are reversible and operate in a single step, so they require no regenerating carrier. There are two main types that differ in the kinetics of the (bio)chemical reaction involved. The configurations for fast kinetics are essentially identical to the previous ones except for the stream contents. In one such configuration (type 1), the sample is injected into a reagent stream and mixing is boosted by means of a coil and/or a point of merging with an additional reagent stream. On the other hand, in the configurations for slow kinetics (e.g. with immobilized enzymes) the analyte–reagent plug is stopped as it reaches the catalyst immobilized at the active microzone in order to make kinetic measurements (signal increments during the stop time). This entails using an electronic or computerized system in order to start and stop the pump at precisely controlled times synchronously with injection. Stopped-flow

systems can be coupled in two main ways to flow-through (bio)chemical sensors: (a) by injecting a single sample aliquot into a conditioning carrier subsequently merged with a reagent stream (type 2) —alternatively, the reagent can be injected and later merged with the sample— and (b) by injecting two microvolumes of sample and reagent simultaneously into a symmetric merging zones configuration (type 3).

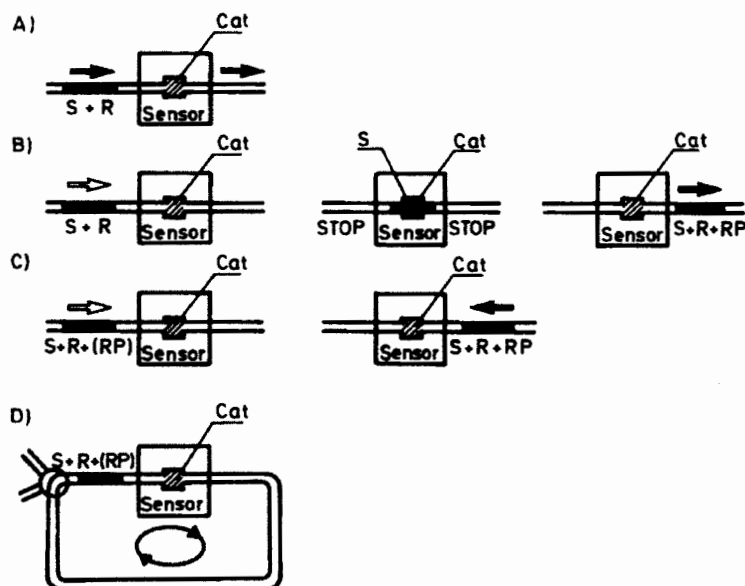


Figure 2.15 — Variants of coupled continuous configurations and sensors accommodating an immobilized catalyst (Cat). (A) Conventional system. (B) Stopped-flow system. (C) Configuration with iterative reversal of the flow direction. (D) Open-closed circuit configuration. Symbol meanings are given in Fig. 2.14. For details, see text.

Kinetic measurements can also be made by using continuous-flow (bio)chemical sensors including a catalyst permanently retained on the active microzone and configurations other than stopped-flow ones. As can be seen in Fig. 2.15, in addition to the configurations described in the previous paragraph (Fig. 2.15.A matches Fig 2.14.1 and Fig. 2.15.B matches Figs 2.14.1 and 2.14.2), one can use two alternatives based on repeated passage of the reaction plug through the detector and involving (a) iterative changes in the flow direction (Fig. 2.15.C) or (b) the use of an open-closed configuration (Fig. 2.14.D). Both provide multipeak

recordings that are used to make the kinetic measurements. A detailed description of the configurations shown in Fig. 2.15 is provided in Section 3.1.

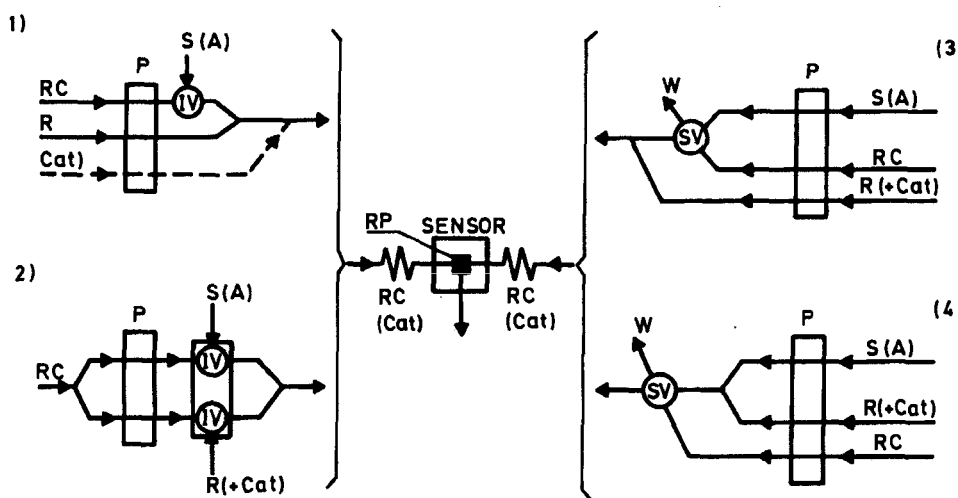


Figure 2.16 — Continuous configurations coupled on-line to flow-through (bio)chemical sensors involving transient immobilization of the reaction product (R) at the active microzone. Symbol meanings are given in Figs 2.12 and 2.14. For details, see text.

Figure 2.16 shows the more frequently used continuous configurations for implementing (bio)chemical *sensors accommodating a temporarily immobilized reaction product* in the active microzone. The product in question is formed in a reaction coil immediately preceding the sensor, even though a reactor containing an immobilized catalyst can also be used for this purpose. This type of sensor integrates two steps (separation and detection) only in the active microzone and inevitably involves two operations (retention and elution of the previously formed reaction product), so it almost always requires a regenerating carrier. It is thus an irreversible-reusable type of sensor. If the reaction product were not removed, the sensor would saturate after the sequential introduction of a small number of samples. There are two possible configurations depending on whether the sample is introduced by injection (types 1 and

2) or aspiration (types 3 and 4); in the former, the regenerating carrier acts as the carrier as well, while in the latter it is inserted after the sample is aspirated. In all these configurations, the reagent solution is inserted by aspiration (types 1, 3 and 4) or injection (type 2). If a catalyst is required to accelerate the primary analytical reaction, it can be incorporated in several ways, namely: (a) by inserting a stream containing it after that of the reagent (type 1) or previously mixed with it; (b) by including it in the reagent stream (types 2, 3 and 4); and (c) by using a solid reactor accommodating it in immobilized form, placed in the reaction coil preceding the sensor. Configuration 2 is more suitable than 1 when the reagent and/or catalyst are scant or expensive. On the other hand, configuration 4 is normally used when the reagent may interact with the regenerating carrier (type 3). The sample volume to be used is dictated by the analyte concentration to be measured and the sensor sensitivity. Configurations 1 and 2 are indicated when no preconcentration is required, while 3 and 4 are especially useful for determining low analyte concentrations since they allow the reaction product to build up at the detector.

It should be noted that Figs 2.12–2.16 do not include every possible type of configuration, as shown in subsequent chapters. Rather, they provide an overview of the wide variety of on-line coupled flow-through sensors and continuous unsegmented-flow analytical configurations.

2.6 REGENERATION MODES

Many flow-through (bio)chemical sensors are irreversible–reusable and operate in two steps, so a description of the different ways in which such sensors can be regenerated, already outlined in the previous section (see Figs 2.12–2.16), is in order. In this context, regeneration can be defined as that operation following passage of the sample through the sensor by which the active microzone is made ready for the next sample by means of a (bio)chemical and/or physico–chemical process. As a result, the continuous signal provided by the sensor is returned to its baseline. Sensor regeneration may involve one or more of the following procedures: (a) flushing any residues of the previously analysed sample from tubes, connectors and the inside of the flow-through sensor itself; (b) removing any potential interferences that might have been retained at the active microzone; (c) eluting the temporarily immobilized species

(analyte, reaction product); (d) conditioning the active microzone, which may entail preparing the immobilized reagent or separation device (*e.g.* a membrane).

Figure 2.17 compares the different ways of regenerating flow-through sensors with the normal procedure for probe sensors: the probe is successively immersed in the sample and buffer solution and removed from it prior to immersion into the next sample, which hinders automated functioning unless a robot station is used (Fig. 2.17.A). On the other hand, on-line coupled flow-through sensors in continuous configurations lend themselves readily to convenient, automated regeneration.

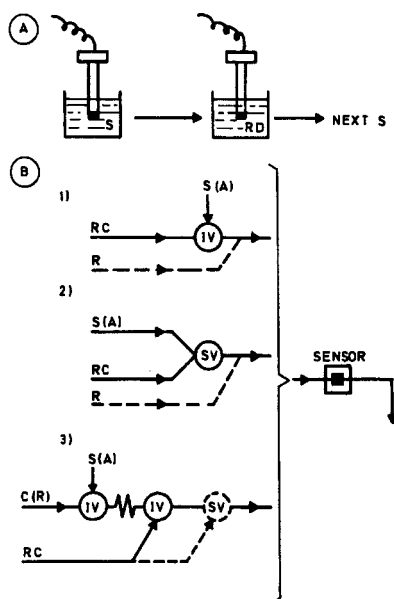


Figure 2.17 — Different procedures for regenerating (A) probe and (B) irreversible-reusable flow-through (bio)chemical sensors. For details, see text.

The simplest procedure for regenerating flow-through sensors (Fig. 2.17.B.1) involves injecting an unusually high volume (between 0.3 and a few millilitres) directly into the regenerating carrier (*e.g.* see [21]). The sensor is placed near the injection valve in order to avoid dispersion (mixing) of the injected macro plug, which reaches the sensor as such or after mixing with a reagent stream. The procedure is most convenient as it is performed immediately after the injected macro plug passes

through the sensor and no extra operation is required since the carrier acts both as such and as regenerator.

One alternative regenerating procedure involves the sequential aspiration of the sample and regenerating carrier (Fig. 2.17.B.2), which requires actuating the switching valve in order to restore the sensor. It allows larger sample volumes to be used in order to raise the analyte concentration at the active microzone when highly dilute samples are to be processed (*e.g.* see [19]).

If the sample must be conditioned, the configuration depicted in Fig. 2.17.B.3) comes in handy since it mixes sample and carrier in a coil before they are transferred to the sensor, which is regenerated at a later stage by introducing the regenerating carrier by injection or aspiration via a switching valve. The carrier is passed through the sensor over a given interval during which the maximum signal provided by the injected sample is recorded.

2.7 TRANSIENT SIGNALS

One essential feature of flow-through sensors is the response they provide via the instrument (detector) to which they are connected or in which they are accommodated. Such a response is typically a transient signal similar to those provide by other systems including FI configurations, (bio)chemiluminescence detectors and electrothermal vaporization atomic absorption spectrometers, but different from the steady-state signals afforded by probe sensors. The shape of such transient signals depends on (*a*) whether the sensor is reversible or irreversible (reusable), (*b*) the type of process taking place at the active microzone (separation, reaction or both), (*c*) which species is immobilized, (*d*) the type of detector used, and (*e*) the operational mode in which the continuous configuration coupled on-line to the sensor is employed.

Figure 2.18 shows the most relevant and/or usual types of transient signals provided by the flow-through (bio)chemical sensors used in the continuous configurations depicted in Figs 2.12–2.16 and the regeneration modes illustrated in Fig. 2.17. The two sequential steps (1 and 2) affecting the sensitive microzone of the flow-through sensor are distinguished.

Reversible sensors afford virtually symmetric transient signals (Fig. 2.18.A) on passage of the sample through the detector (*e.g.* see [15]). Such is the case with sensors involving a permanently immobilized

catalyst and a fast reaction (Fig. 2.14.1) or when the immobilized reagent requires no regeneration and the sample is injected into a conditioning carrier (Fig. 2.13.1).

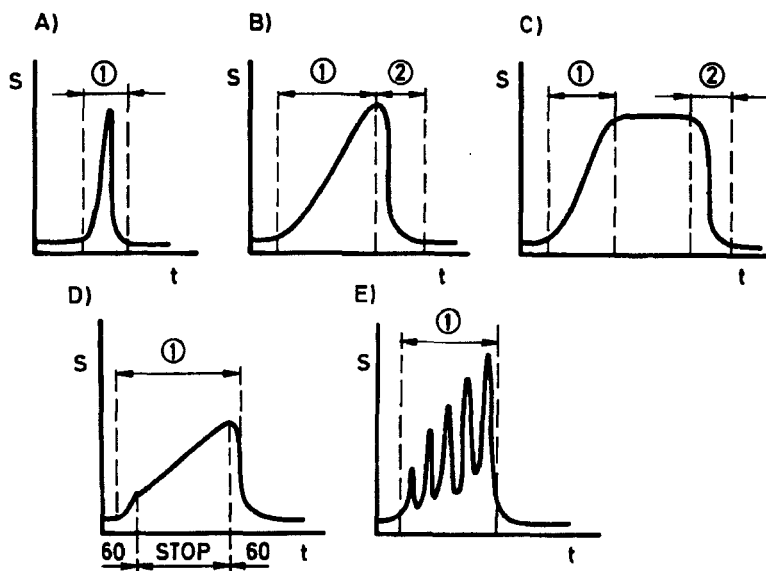


Figure 2.18 — Standard signal–time profiles provided by flow-through (bio)chemical sensors from ordinary (A–C) and kinetic measurements (D,E). Numbers 1 and 2 denote passage of sample and regenerating carrier, respectively. For details, see text.

Figure 2.18.B shows the transient signal obtained by injecting a macro volume of sample into a regenerating carrier that transfers it to the detector, which is immediately followed by elution and regeneration (*e.g.* see [21]). An identical signal–time profile is also obtained if the sample and regenerating carrier are introduced sequentially (*e.g.* see [19]). Steps 1 and 2 involve passage of the sample and regenerating carrier, respectively (Figs 2.17.B.1 and 2.17.B.2). These profiles are similar to one another but different from those provided by ordinary FIA systems, which exhibit a more abrupt start and a more gradual return to the baseline. The signals afforded by flow-through sensors also resemble those provided by probe sensors (Fig. 2.17.A): they rise as the probe is immersed in the sample and fall as it is removed from (reversible

sensors) or immersed in a regenerating solution (irreversible–reusable sensors).

The initial and final portions of the signals obtained by injecting the sample into a conditioning carrier for transport to the sensor (Fig. 2.18.C) are similar to those of the previous signals (Fig. 2.18.B). The central portion is plateau-shaped and arises from passage of the carrier during the time elapsed between the end of the process (reaction and/or detection) in the sensitive microzone and arrival at the microzone of the regenerating carrier introduced at a later stage by injection or aspiration via a switching valve (see Figs 2.12.3, 2.12.4, 2.13.3, 2.16.3, 2.14.6).

Stopped-flow methods based on the use of an immobilized catalyst (Figs 2.14.2 and 2.14.3) provide signal profiles that exhibit the typical rise observed during the stop time (Fig. 2.18.D) (*e.g.* see [27]). Repeated passage of the same sample plug through the sensor containing the immobilized catalyst (Figs 2.15.C and 2.15.D) gives rise to a multipeak recording per injected sample (Fig. 2.18.E) (*e.g.* see [38,39]).

2.8 MEASUREMENT MODES

The transient signals provided by flow-through (bio)chemical sensors can be processed in various ways in order to draw information that can be directly related to the analyte concentration in the sample. Figure 2.19 shows the more frequently used approaches in this respect, classified according to whether they rely on direct (A) or kinetic measurements (B).

The most common procedure (Fig. 2.19.A) involves measuring the transient signal provided by a reversible or irreversible–reusable sensor at the maximum and the plateau obtained by injecting the sample into a non-regenerating carrier.

Kinetic measurements are based on signal increments over preset intervals and have the advantage of their relative rather than absolute nature, which avoids interferences from the sample matrix. Figure 2.19.B shows the different variants of kinetic measurements in this context, which depend on the type of sensor and coupled continuous configuration used. The most immediate variant involves halting the flow over an interval Δt when the sample plug reaches the detector (Fig. 2.19.B.2), where the (bio)chemical reaction is allowed to develop while the product of interest is monitored simultaneously. The other two variants

warrant more detailed discussion on account of their innovativeness and great interest and potential.

Kinetic *in situ* concentration methods (Fig. 2.19.B.1) rely on measurements of the slope of the initial portion of the transient signal, which arises from passage of the sample plug through the detector (Fig. 2.18.B) (*e.g.* see [19]). The signal rises as the analyte accumulates on the active microzone. The signal increment over a preset interval is proportional to the analyte concentration in the sample. In common with every kinetic method, *in situ* concentration methods offer substantially increased selectivity. In addition, they avoid the need to wait for the signal maximum to be reached (*i.e.* for the whole injected or aspirated sample macro plug to pass through the reactor) since the signal increment over a preset interval soon after injection is more than adequate for measurement purposes; in this way, the sample throughput can be doubled or trebled as a result. Microprocessors for signal acquisition and processing make this an affordable choice for routine analyses of vast numbers of samples. In addition, kinetic *in situ* concentration methods avoid the two most severe shortcomings of conventional preconcentration methods, *viz.* their slowness and the need to use large sample volumes.

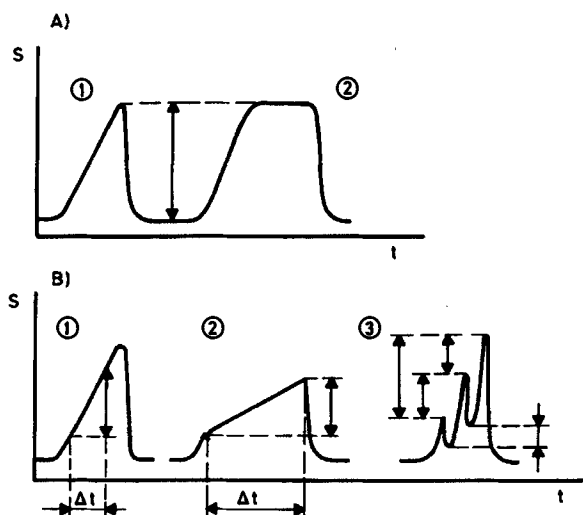


Figure 2.19 — Types of measurements available on the transient signals provided by flow-through (bio)chemical sensors. (A) Ordinary measurements. (B) Kinetic measurements. For details, see text.

The multi-peak recordings obtained by passing the sample plug many times through the detector (Fig. 2.14.C) or using an open-closed circuit (Fig. 2.15.D) can be used to draw valuable information from various types of measurement (Fig. 2.19.B.3) (*e.g.* see [38,39]). Thus, signal increments can be measured between peak maxima and minima at fixed intervals, so they can also be considered kinetic measurements. Alternatively, individual kinetic measurements can be added up in order to boost the sensitivity of the flow-through sensor concerned, which usually arises from inclusion of a permanently immobilized reagent in the active microzone.

2.9 THE ROLE OF KINETICS

Notwithstanding the impressive developments in (bio)chemical sensors over the past decade, little emphasis has been placed on their kinetic connotations, which are only considered when the sensor in question provides a "slow response". It is therefore a priority to devote generous efforts to studying the kinetic undertones of sensors in order to expand their development and applications. However, while kinetics is the key in broad terms, it is even more significant to flow-through sensors on account of their dynamic character (*e.g.* see [40]).

One temporal concept to be borne in mind in this context is whether the (bio)chemical reactions and mass transfer separations taking place at the active microzone (one or both of which, by definition, take place simultaneously with detection) are simultaneous or sequential relative to each other. Whether such processes take place at the same or a different time has a marked effect on the sensor performance and type of transient signal obtained.

In dealing with the mechanism of action (and response) of a flow-through (bio)chemical sensor, one should consider three different types of kinetics (Fig. 2.20), namely:

(a) *Physical kinetics*, which arises from the intrinsic dynamic character of flow-through sensors and is essentially related to the transfer of sample, reagent and regenerating carrier to the sensor microzone. This type of kinetics is chiefly governed by the geometric characteristics of the coupled configuration and sensor, as well as their hydrodynamic features (flow-rates). Physical kinetics is also primarily responsible for

the transient character of the signals afforded by (bio)chemical sensors (Fig. 2.18).

(b) *Physico-chemical kinetics*, which is basically related to separation processes that take place at the sensor microzone: transfers of analytes or reaction products across separation membranes to the surface or inside of a sorbent material until a partitioning equilibrium is reached. This type of kinetics comes into play twice in irreversible-reusable sensors, viz. during passage of both the sample containing the analyte or reaction product and the regenerating carrier. On the other hand, it only affects one step in the application of reversible sensors. This type of kinetics is strongly influential on the profile of the transient signal obtained (particularly on its onset and tail). On the other hand, this component of the overall sensor response is essentially affected by such factors as the diffusion coefficient; pore size; membrane thickness, surface activity and location; flow-rates and temperature.

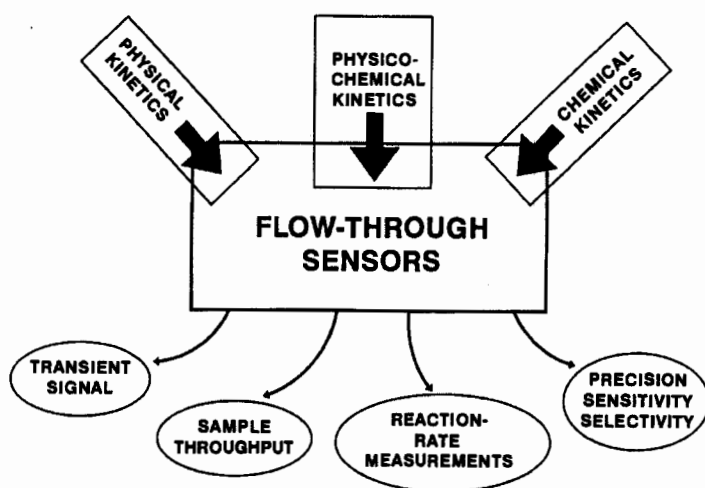


Figure 2.20 — Kinetic aspects of flow-through (bio)chemical sensors and analytical implications.

(c) *Chemical kinetics*, which is associated with the chemical reaction that takes place simultaneously with detection and sequentially or simultaneously with a separation process at the active microzone. This

type of kinetics is particularly significant when the catalyst is the immobilized species as it determines the most suitable continuous configuration for on-line coupling to the sensor (see Figs 2.15 and 2.16). Alternatively, the catalyst can be included in the reagent solution or immobilized in a prior mini-reactor (Fig. 2.16).

With the exception of flow-through sensors used for reaction rate measurements, the processes taking place at the active microzone of a flow-through sensor (transport, separation and reaction) must be rapid enough for the transient signal to be as short-lived as possible. The sample, reagent, regenerating carrier and conditioner flow-rates used must be compatible with the kinetics of the chemical and physico-chemical processes occurring at the sensor. As a rule, the rate of the process is determined by those factors governing mass transfer across a membrane or liquid-solid interface. It is therefore essential that any separation taking place at the active microzone be as rapid and efficient as possible. In this way, the flow-rates used in the continuous configuration can be safely increased and the sample throughput boosted as a result.

The three above-mentioned types of kinetics also influence other aspects of sensor performance (Fig. 2.20). Thus, the signal-time profiles they provide are critically dependent on the kinetics of the processes involved; for example, if the sensor regeneration is rather slow, baseline restoration is much too slow. As noted earlier, a slow chemical kinetics can be used to perform reaction rate measurements.

On the other hand, it should be emphasized that such basic analytical properties as precision, sensitivity and selectivity are influenced by the kinetic connotations of the sensor. Measurement repeatability and reproducibility depend largely on constancy of the hydrodynamic properties of the continuous system used and on whether or not the chemical and separation processes involved reach complete equilibrium (otherwise, measurements made under unstable conditions may result in substantial errors). Reaction rate measurements boost selectivity as they provide differential (incremental) rather than absolute values, so any interferences from the sample matrix are considerably reduced. Because flow-through sensors enable simultaneous concentration and detection, they can be used to develop kinetic methodologies based on the slope of the initial portion of the transient signal, thereby indirectly increasing the sensitivity without the need for the large sample volumes typically used by classical preconcentration methods.

2.10 REQUIREMENTS FOR PROPER SENSOR PERFORMANCE

For a given flow-through (bio)chemical sensor to be useful for solving real analytical problems, it must meet the indispensable or desirable requirements discussed in Chapter 1. One should bear in mind that many reported (bio)chemical sensors have some deterrent shortcomings (*e.g.* a slow response, a high sophistication, irreversibility, too short duration, irreproducibility, ease of contamination) that make them useless for routine applications in practice. Flow-through sensors should therefore meet four essential requirements for proper performance, namely:

(a) They should be fully reversible (or readily and expeditiously regenerable otherwise). Hence single-use (*e.g.* probe and drop planar) sensors are of very little service here. Flow-through sensors including an immobilized catalyst and ion-selective electrodes accommodated in flow-cells are typically reversible sensors. As a rule, most of them must be regenerated after one or more samples have been passed. When the immobilized reagent is consumed in the process, the sensor is actually irreversible but can be used for over 50 determinations—it is thus usable for practical purposes—in many instances.

(b) The type of immobilization used should be suited to each particular sensor. With permanent immobilization (reagent or catalyst), the physico-chemical linkage or chemical bond of the immobilized species to the support must be highly stable under the sensor operational conditions. With sensors based on a transiently immobilized analyte or reaction product, chemical or physico-chemical retention must be very strong in order to avoid sweeping by the sample or conditioning carrier, but also be readily and rapidly overcome on passage of the regenerating carrier through irreversible-reusable sensors.

(c) As noted earlier, the physical, physico-chemical and (bio)chemical kinetics inherent in on-line coupled biosensors and continuous configurations must be fast enough for the sensor to perform properly.

(d) The active microzone and sensing system used must be mutually compatible, *i.e.* the detector must be responsive to changes in the microzone, which in turn must not alter the detector functioning. Such compatibility reflects in the obtainment of a stable baseline (close to the zero signal level) in the absence of sample and a perceptible rise on its passage through the detector.

The above four requirements vary somehow with the type of flow-through sensor and detection system used. There are other, specific requisites, discussion of which is beyond the scope of this introductory chapter. A more detailed description of the optimal operational conditions for flow-through sensors is provided in subsequent chapters.

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3

Flow-through sensors based on integrated reaction and detection

3.1 INTRODUCTION

Flow-through sensors integrating detection and a chemical or biochemical reaction rely on immobilization in the probe proper or the flow-cell (or a special housing included in it) of a species intended to take part in or catalyse the reaction by which the analyte, *viz.* the catalyst or reagent, is measured, according to which the sensors described in this Chapter are divided into two broad categories.

Sensors based on an immobilized catalyst include some of great interest, wide use and a high potential for future developments. They rely on a biological material (usually an enzyme, but also occasionally a different biomolecule or a tissue) to facilitate development of the analytical reaction in a fairly short time. The significance of these so-called "biosensors" is clearly reflected in the large number of books published on them in the last few years [1–9]. They make the larger group of the two dealt with in this Chapter as they encompass a wide variety of biocatalysts and detection techniques including optical (absorptiometric, luminescence), electroanalytical (amperometric, potentiometric) and —to a lesser extent— miscellaneous (thermal, mass, enthalpimetric, acoustic) alternatives (see Section 3.2.1). While less numerous, sensors based on non-biological catalysts are also of some interest (see Section 3.2). Sensors based on immunoassays are dealt with in Section 3.3.

Unlike sensors based on an immobilized catalyst, those using an immobilized reagent can be classified according to whether the reagent is consumed (Section 3.4.1) or regenerated in the process (Section 3.4.2).

In addition to the above basic types of sensors, this Chapter deals with another, smaller group of devices where the reagent is regenerated in the flow-cell itself (Section 3.5).

3.2 FLOW-THROUGH SENSORS BASED ON AN IMMOBILIZED CATALYST

Using a catalyst immobilized on the sensing surface or the flow-cell (or a special built-in housing) is the best approach to sensing when the measuring reaction is integrated with detection since the catalyst behaves reversibly. The way in which the catalyst acts in a (bio)chemical reaction allows development of reversible sensors that can in theory be used an unlimited number of times. In practice, however, constraints imposed by the properties of the sample and/or the medium where the derivatization reaction involved takes place, in addition to the nature of the catalyst itself, limit the serviceability of sensors to a greater or lesser number of uses —or even a single use in disposable sensors.

One essential difference between flow-through sensors based on an immobilized catalyst arises from the nature of the catalyst, *viz.* biochemical (usually an enzyme) or chemical (normally an inorganic species). The latter are much less numerous and frequently used.

One other difference lies in the type of detection technique used, which dictates the flow-cell design. Thus, a distinction can be made in this respect between optical (absorptiometric, luminometric) sensors, which make measurements of the bulk solution where the flow-cell is immersed, and electroanalytical (amperometric, potentiometric) sensors, where measurements are based on phenomena occurring at the electrode–solution interface.

3.2.1 Enzyme sensors

The flow-through sensors described in this Section comply essentially with the definition of biosensor. This word, like every term used to designate devices of scientific and popular note, has been the object of a number of definitions of both generic and specific scope. In a broad sense, a biosensor is any instrument or technique that measures biomolecules. In stricter terms, Rechnitz defines a biosensor as "a device that incorporates a biochemical or biological component as a molecular recognition element and yields an analytical signal in response to biomolecules" [10]. In between these two

extreme definitions, a sensor can be regarded as "a system of two transducers, biochemical and physical, in intimate contact with each other, which relate the concentration of an analyte to a measurable electrical signal". As a rule, a biosensor is a sensor that enables measurements of biological species, one in which both the species that facilitates recognition and the analyte are of biological nature, or one in which only the recognition element is biological, depending on which definition is adopted.

There is the widespread feeling that biosensors have been overpromoted. In fact, they have suddenly started to be praised as providing solutions to a wide variety of scientific and societal problems. To a great extent, such overpromotion has resulted from overoptimistic market projections by research bodies with little understanding of the tremendous problems still facing biosensors. Such projections are reported as facts in the media, which raises unrealistic expectations.

One other false impression about activity in the biosensor field is provided by overall publication rate counts. If reviews, meeting proceedings and other re-publications, routine application studies and duplicated efforts (some biosensors are seemingly reinvented as often as every couple of years) are subtracted, the actual amount of original work in this field is probably not very large and significantly concentrated in a few major laboratories. Even the casual reader of the biosensor literature soon realizes that the scientific level ranges from rudimentary to highly sophisticated, with a distribution skewed rather to the former.

On the other hand, the international scope of biosensor research is quite a positive indicator. The relative low equipment costs involved and the wide availability of biological materials has facilitated access of not so wealthy or industrialized countries to biosensor research, which ensures a healthy diversity of approaches and leaves the field open to fresh and, perhaps, radical new ideas.

On the technical side, the greatest hurdles to a wider use and appreciation of biosensors are biocompatibility and *in situ* calibration. Developing biosensors that function well in the research laboratory is one thing and making them capable of performing properly in a realistic bioanalytical environment is quite another. Legislation aside, no biosensor operating satisfactorily inside a living being —except for a short period of time— has so far been developed. In fact, reliable sterilization is the minimal requisite for *in vivo* applications, yet many potentially usable biosensors fail to meet it.

Calibration is a particularly critical problem in long-term biosensor applications involving implantation or unattended operation. Thus, biosensors including biological or biochemical components are not particularly stable over time, so they are prone to result in significant fluctuations. Also, seemingly identical sets of sensors typically give rise to irreproducibility problems. Finally, such sensors age at different rates and may reach a failure threshold sooner than expected from conventional laboratory estimations—indeed, the usually limited lifetime of a biosensors is still an imposing hurdle for a number of major applications that impedes wider spread.

The state of the art in flow-through sensors based on immobilized enzymes is a result of intensive research activity, particularly in the 1980s and early 1990s. Studies in the field have been concerned with both the development of new types of supports [11] and alternative immobilization techniques [11–13]. The wide used of immobilized enzymes in continuous-flow systems (mainly in flow injection analysis, FIA) [15–29] has facilitated understanding of the behaviour of these biochemical materials in dynamic systems. The high flexibility of these systems for sample manipulation prior to detection is the basis for their use in complex systems involving multi-stage conditioning treatments (*e.g.* dialysis, pre-electrolysis, an enzyme reaction preceding detection [30]) assuring reliable subsequent quantitation or enabling multi-determinations [31].

These preliminary developments have relied on either metabolic enzyme reactions (*viz.* those where the substrate is consumed and a product is formed as a result) and bioaffinity reactions (*viz.* those that are followed via electron density changes). This Section discusses sensors based on immobilized enzymes and both types of reaction: metabolic and bioaffinity.

Given the wide variety of available flow-through sensors using an immobilized enzyme, they are dealt with according to the type of detection involved: optical or electroanalytical. Each sensor group is in turn divided according to various criteria.

3.2.1.1 Flow-through optical biosensors

Flow-through optical sensors bearing one or more immobilized enzymes at their sensing microzone can be classified according to the type of physical relationship between the microzone and the detection system or instrument used into those using fibre optics (photometric and luminometric) and those integrating a biochemical reaction and detection (usually photometric).

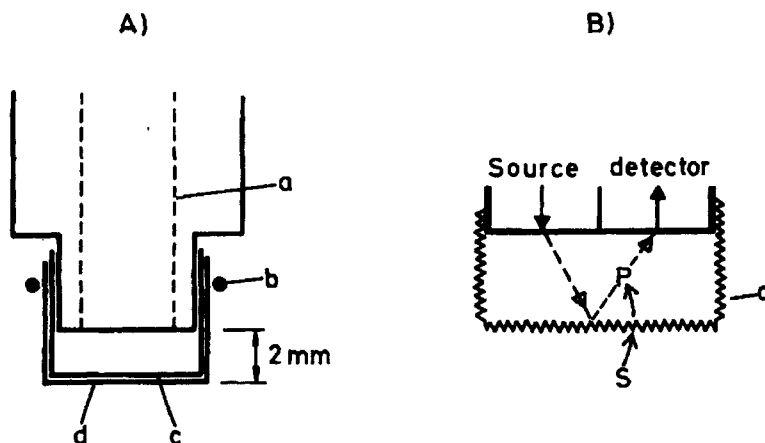


Figure 3.1 — (A) Configuration of p-nitrophenyl phosphate biosensor: (a) common end of bifurcated bundle; (b) retaining O-ring; (c) inner nylon mesh with enzyme; (d) outer nylon mesh (not drawn to scale). (B) Processes occurring at the biosensing tip: a enzyme/scatter layer; S enzymatic substrate; P light absorbing product. (Reproduced from [34] with permission of the American Chemical Society).

3.2.1.1.1 Fibre optic-based flow-through optical biosensors

The dramatic advances in fibre optic development in the last decade have promoted construction of sensors where radiation, whether emitted, transmitted or reflected, is conducted from the sample to the detection system. The wide variety of available optical waveguide types (solid rods, hollow cylinders, micro-planar geometries) has been used with varying success in sensor development.

Guilbault *et al.* [32] developed a *photometric* fibre-optic device for the colorimetric determination of total and free cholesterol based on a previous design using two reactors accommodating two enzymes (cholesterol oxidase and sterase) co-immobilized in a pre-activated membrane. The membrane was fixed around the common end of a bifurcated fibre-optic bundle. The enzymatic reaction took place on immersion of the fibre and membrane into the sample cell containing diammonium 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate), peroxidase and the cholesterol sample. The cholesterol concentration was related to the intensity of colour development with kinetic evaluation and the results of one measurement were obtained within 2 min [33]. The expeditiousness of the enzymatic and non-enzymatic steps involved

make the sensor suitable for use under continuous-flow conditions provided the derivatizing reagent and sample are mixed prior to the flow-cell, where the flow can be stopped to make reaction-rate measurements.

In 1985, Arnold developed a biosensor for the determination of *p*-nitrophenylphosphate using immobilized alkaline phosphatase and reflectance measurements of the resulting *p*-nitrophenoxide [34]. Figure 3.1.A depicts the fibre-optic biosensor configuration used, which included two sheets of nylon mesh held at the common end of the fibre bundle. The enzyme was immobilized on the inner nylon membrane and the outer membrane functioned to scatter incident radiation. Alkaline phosphatase was immobilized by covalent bonding to the nylon, which was altered in order to provide a suitable functional group. A small piece of nylon webbing (pore size 5 μm) was secured around a glass rod with the aid of a nylon thread. This nylon net was placed in a simmering dimethyl sulphate solution and allowed to stand for 5 min. The treated nylon net was then immersed in a test tube containing freshly distilled, anhydrous methanol for 30–40 s, followed by a second immersion in fresh, anhydrous methanol for 1 min. After this treatment, the nylon was released from the rod and placed in a 50-ml solution of 0.5 M lysine at pH 9.0 for 2 h. The net was then thoroughly washed with 1.0 M NaCl. At that point, a small portion of the membrane was tested for the presence of amino groups by placing a small amount of 2,4,6-trinitro-

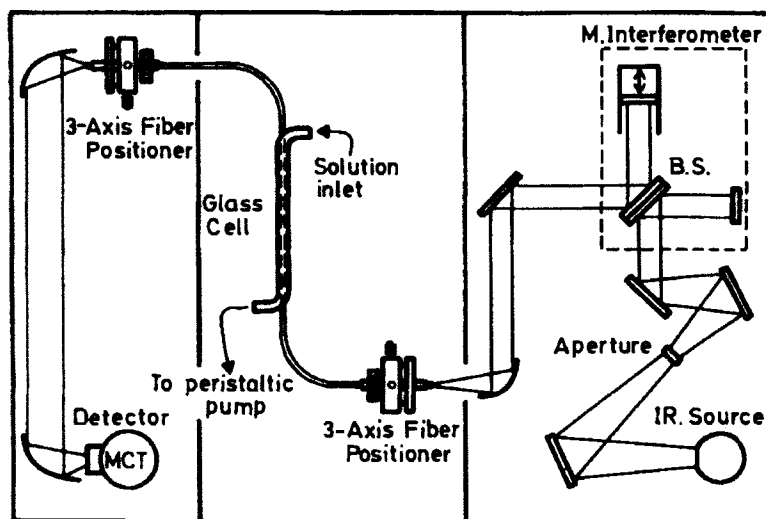


Figure 3.2 — Optical arrangement of chemical IR sensor.

benzenesulphonic acid on it and checking for the appearance of the characteristic yellow–orange colour. Next, the whole membrane was immersed in a 12.5% glutaraldehyde solution containing 0.1 M borate at pH 8.5 for 45 min. Following a second wash with 0.1 M NaCl, the treated net was placed in an alkaline phosphatase solution consisting of 0.1 M phosphate buffer of pH 7.0 and *ca.* 100 IU of the enzyme at 4°C overnight. Figure 3.1.B schematizes the processes taking place at the fibre optic surface. As the enzyme substrate diffused from the bulk solution into the enzyme layer, the enzymatic reaction was catalysed and the spectrophotometrically active product generated. The product absorbed a given fraction of back-scattered radiation, so a decrease in the light intensity was observed as it accumulated.

Infrared transparent fibres were recently developed for telecommunication, laser power transmission for surgical purposes, military uses and spectroscopy. The constituent materials are usually chalcogenide glasses, in addition to fluoride glasses, heavy metal oxides and assorted crystalline materials on account of their superior optical and mechanical properties. The earliest attempts of Kellner *et al.* at constructing an infrared-attenuated total reflection–IR–ATR-based glucose sensor, which involved using an enzyme layer immobilized via 3-aminopropyltriethoxysilane (3-APTS) and glutaraldehyde, led to a stable glucose oxidase film deposited on a germanium crystal thinner than the penetration depth of the IR radiation ($< 0.5 \mu\text{m}$) [35] and the subsequent development of a fully reversible *IR fibre optic biochemical sensor* for glucose [36]. One third of the chalcogenide glass fibre (10 cm) was used as an intrinsic sensing element and successfully derivatized. The quality control of the first chemical process on the uncoated fibre surface, silylation, was carried out by using scanning electron microscopy, secondary ion mass spectrometry and infrared microscopy. Homogeneity of the 3-APTS layer was checked by analysing the silicone peak intensity. Glucose oxidase was immobilized optimally after removing the protective polyamide coating of the fibre surface, which was coupled to a commercially available FT–IR spectrophotometer by means of specially designed focussing optics (Fig. 3.2). Glucose solutions were pumped through a glass cell of 0.5 mL and continuous *in situ* measurements were provided by the spectrophotometer in real time. Glucose could thus be detected in the lower biological range by measuring the absorbance at 1153 cm^{-1} of the gluconic acid produced by oxidation of the glucose, catalysed by enzyme covalently bonded to the fibre surface. The sensor is representative of the

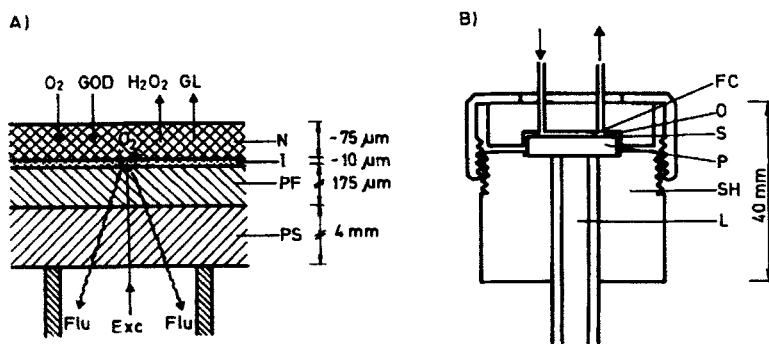


Figure 3.3 — (A) Schematic diagram of flow-through cell for glucose and alignment of the fibre end: FC flow-through chamber; O O-ring; S sensing platelet; P Plexiglas disc; SH stainless-steel housing; L light guide. (B) Cross-section through the sensing layer of the fibre optic glucose sensor: PS Plexiglas support; PF polyester film; I indicator layer (decacyclene dissolved in silicone); N nylon membrane with enzyme immobilized on its surface. Arrows indicate the diffusion processes involved (Glu, glucose; GL, gluconolactone). The directions of the exciting light (Exc) and fluorescence (Flu) are also shown. (Reproduced from [37] with permission of the Royal Society of Chemistry).

strongly enhanced capabilities of chemically modified IR fibre optics for use in miniature biosensors.

There are several *luminescence* flow-through sensors based on both fluorescence quenching phenomena and bioluminescent reactions.

Several research groups have developed *fluorimetric sensors* based on measurement of the oxygen uptake during oxidase-catalysed reactions via fluorescence quenching. Thus, Wolfbeis *et al.* constructed a fibre-optic sensor for glucose based on the dynamic quenching of the fluorescence of a dye by molecular oxygen. The sensing layer of the fibre optics was prepared as follows: glucose oxidase was immobilized covalently on a nylon immunoaffinity membrane via chemically activated carboxyl groups, while the dye was dissolved in a very thin silicone membrane placed beneath the enzyme layer as shown in the cross-sectional view of the sensing layer in Fig. 3.3.A. Figure 3.3.B shows how the fibre end was aligned with the flow-cell. The amount of oxygen consumed in the enzyme-catalysed reaction was measured via the increase in the dye fluorescence relative to the signal obtained in the absence of substrate. Measurements were made in flowing-air saturated solutions containing a phosphate buffer [37].

Schmid *et al.* used the same principle to develop sensors to be incorporated into FI systems for the determination of ascorbic acid in fruit juices [38] and that of lactic acid in dairy products [39]. The membrane used in both applications consisted of decacyclene dissolved in silicone rubber that was treated similarly as the membrane in glucose sensors (Fig. 3.4.B). The oxygen optrode was coated with a sheet of carbon black as optical insulation in order to protect it from ambient light or intrinsic sample fluorescence. Ascorbic acid oxidase or lactic acid oxidase was immobilized by adsorbing it onto carbon black and cross-linking it with glutaraldehyde. The FI system automatically buffered and diluted the food samples, thereby protecting the biosensor from a low pH and interferences.

In 1988, Müller *et al.* developed a special fluorimetric flow-through biosensor for monitoring growth of *Saccharomyces cerevisiae* in aerobic fermentation cultures containing suspended and immobilized cells in order to investigate the influence of cell immobilization [40]. The experimental set-up used for this purpose is shown in Fig. 3.5. Yeast cells were placed in the reactor and the culture fluorescence was measured by connecting the reactor to the fermenter via a recirculation loop. The reactor was sterilized together with the fermenter and the fluorescence detector was then interfaced to it via a sensor port. The reactor and sensor were separated by a quartz window. This monitoring system was found to perform brilliantly, as reflected in the lower ethanol consumption by immobilized yeasts relative to suspended cells. The reduced rate of oxidative decomposition may have been the result of oxygen mass-transfer constraints.

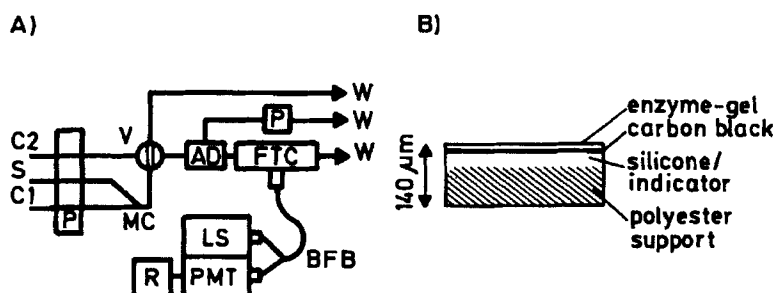


Figure 3.4 — (A) FIA manifold for the determination of L-lactic acid in milk products: P peristaltic pump; C carrier; S sample; V injection valve; AD air damper; FTC flow-through cell; W waste; BFB bifurcated fibre; LS light source; PMT photomultiplier tube; R recorder. (B) Cross-section of lactic acid optrode. (Reproduced from [39] with permission of VCH Publishers).

Light production in some biochemical reactions (bioluminescence) has been recognized as a powerful tool for biochemical and clinical analyses. Emitted light can be measured with a high sensitivity, so very low detection levels can indeed be achieved.

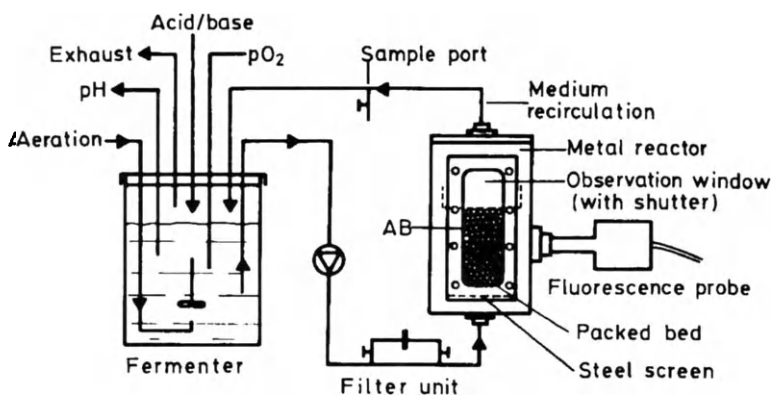


Figure 3.5 — Reactor system: fermenter, 1000 mL; metal reactor, 135 mL; reactor material, stainless steel (VIIA); AB alginate beads. (Reproduced from [40] with permission of Elsevier Science Publishers).

There are relatively few fibre-optic *bioluminescence flow-through sensors*. The early designs of Blum *et al.* for measuring NADH, ATP and H_2O_2 used an enzyme membrane that was brought into contact with one end of a glass-fibre bundle via a screw cap (Fig. 3.6.A). The other end of the bundle was placed near the window of a luminometer photomultiplier tube. Luciferase + oxidoreductase and firefly luciferase were immobilized on PALL^R for the determination of NADH and ATP, respectively, while horseradish peroxidase was immobilized on a pre-activated nylon membrane for determining hydrogen peroxide [41–44]. The scope of application of this sensor was expanded by its proponents by co-immobilizing suitable dehydrogenases for the microdetermination of ethanol, sorbitol and oxaloacetate [45]. They also developed a new methodological approach involving the use of a multi-purpose fibre-optic sensor for the microdetermination of ATP and NADH [46] based on the co-immobilization of luciferase + oxidoreductase and firefly luciferase, which allowed the alternate determination of the two analytes without the need to replace the sensing element of the biosensor simply by chaining the co-reactants in the medium. By using an appropriate

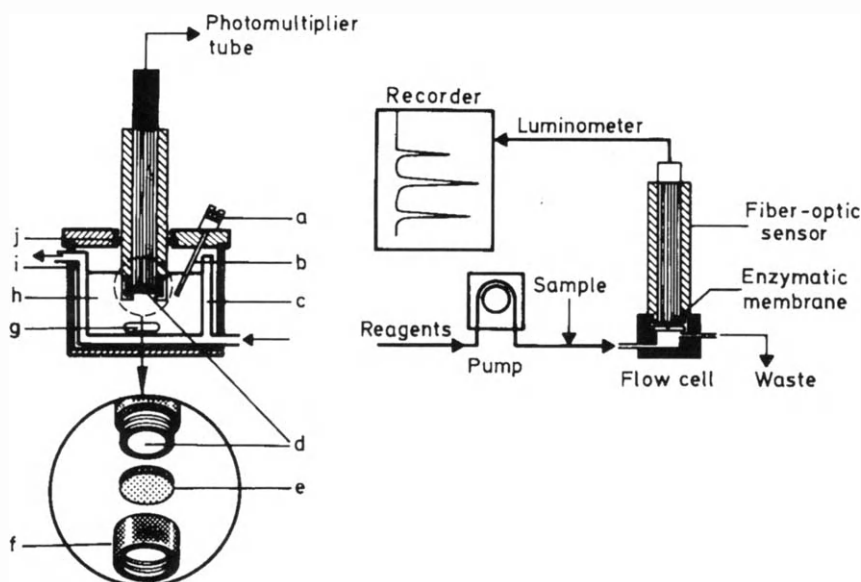
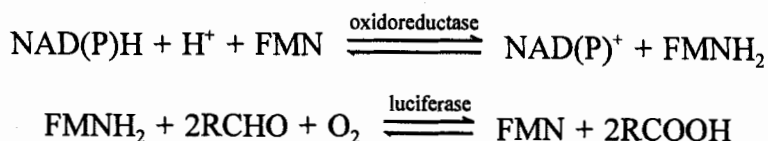


Figure 3.6 — (A) Fibre-optic biosensor system: a septum; b needle guide; c thermostated reaction vessel; d fibre bundle; e enzyme membrane; f screw cap; g stirring bar; h reaction medium; i black PVC jacket; j O-ring. (B) Continuous-flow fibre-optic sensor system for the bioluminescence determination of NADH. (Reproduced from [41] with permission of Marcel Dekker, Inc.)

flow-cell, a sensor of this type was developed for the determination of NADH in a dynamic single-channel system such as that shown in Fig. 3.6.B. The carrier-reagent used to inject the sample contained bovine serum albumin, dithiothreitol and flavin mononucleotide (FMN). On contacting the enzymes immobilized on one end of the fibre optic bundle, the analyte reacted in two steps, namely:



Up to 25 samples/h could be measured in this way with no carryover. Also, no loss of activity was observed after 150 determinations carried out over a 3-day period [47].

3.2.1.1.2 Integrated flow-through optical biosensors

The most immediate advantage of integrating sensors in a measuring instrument is the increased sensitivity arising from direct irradiation by the light source, which avoids the typical energy losses associated with transport along optical fibres. Other, additional advantages result from the simpler, more inexpensive experimental set-up required; in fact, any photometer or spectrofluorimeter is fit for the purpose (in many cases, a conventional flow-cell is more than adequate).

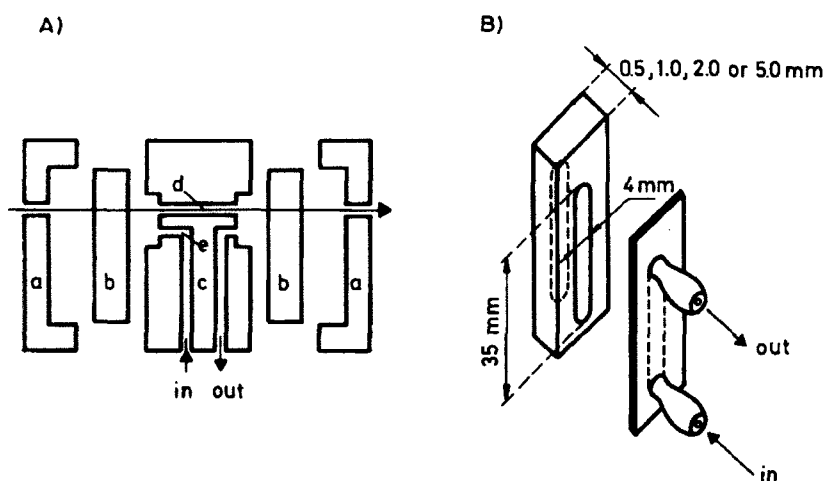


Figure 3.7 — (A) Cross-sectional view of the McPherson stopped-flow mixer unit. The outer aluminum housing (*a*) and quartz windows (*b*) are press-fitted with three bolts. Mixing occurs at *e*, where the streams meet at 90° to each other; one stream is in the figure plane and the other normal to it. The immobilized enzyme reactor is placed inside *d*. With the reactor in place, the observation cell is 1.75 cm in length. The dashed arrow represents the lightpath inside the cell. (B) Flow-cell used to accommodate enzymes on CPG. (Reproduced from [48] and [49] with permission of Elsevier Science Publishers).

One of the earliest and most straightforward *integrated photometric flow-through sensors* was developed by Thompson and Crouch in 1982 by altering the flow-cell of a commercially available stopped-flow module (Fig. 3.7.A). The sensor was disassembled and a 1.75-cm section of a nylon tube of 0.1 cm ID containing lactate dehydrogenase bound to its inner walls was fitted tightly inside it, after which the sensor was reassembled. In broad terms, the system operated as follows: the two syringes of the stopped-flow module

were filled with distilled, de-ionized water, the water was pushed through the sensor and the flow was stopped. This sequence was repeated six times to flush the nylon reactor thoroughly. Next, the syringes were loaded with the reagents, which were pushed through the mixer and sensor, and the flow was again stopped. This second sequence was repeated five times to assure quantitative (1+1) mixing of the β -NAD and lactate solutions. All these steps were repeated in each experiment, which thus required 4 mL of water and 1.85 mL of each reagent. The blank used was a β -NAD working-strength solution mixed with distilled water. Because the reaction takes place under static conditions, its kinetics was controlled by diffusion and the inherent enzyme reaction rate [48].

One more recent sensor design uses an FI configuration coupled to an ordinary photometric detector. Pre-activated CPG was glued to the inner walls of the disassembling flow-cell used (Fig. 3.7.B), which were then glued to each other, after which alcohol dehydrogenase was immobilized by recirculation for 4 h. Kinetic measurements allowed the system to be applied to wine and whole blood samples. The experimental procedure used involved the simultaneous injection of the sample and co-enzyme into two buffer streams that were merged prior to the flow-cell, where the sample/co-enzyme mixture was halted by stopping the peristaltic pump via which the system dynamics was controlled. A computer was employed to acquire absorbance–time data pairs during the stop time that were subsequently processed and interpolated on a calibration graph in order to calculate the ethanol concentration in the different types of sample [49].

The previous flow-cell was used in conjunction with various FI approaches to the development of photometric reaction-rate measurements involving immobilized enzymes [50] to compare the different ways in which reaction-rate methods can be implemented by using flow-through biosensors and both ordinary and special flow injection manifolds. The three approaches illustrated in Fig. 3.8.A allow implementation of such kinetic methods, with (Fig. 3.8.A.I) or without stopping the flow (Figs 3.8.A.II and 3.8.A.III). In the former case, sample injection is synchronized with stopping of the peristaltic pump in order to acquire data that are converted into a recording such as that shown in Fig. 3.8.B.I, which is used to make absorbance increment measurements over a preset interval. The main difference between the approaches depicted in Figs 3.8.A.I and 3.8.A.II lies in the use of a programmable peristaltic pump in the latter to reverse the flow direction at preset intervals [51] in such a way that the sample–reagent plug is passed

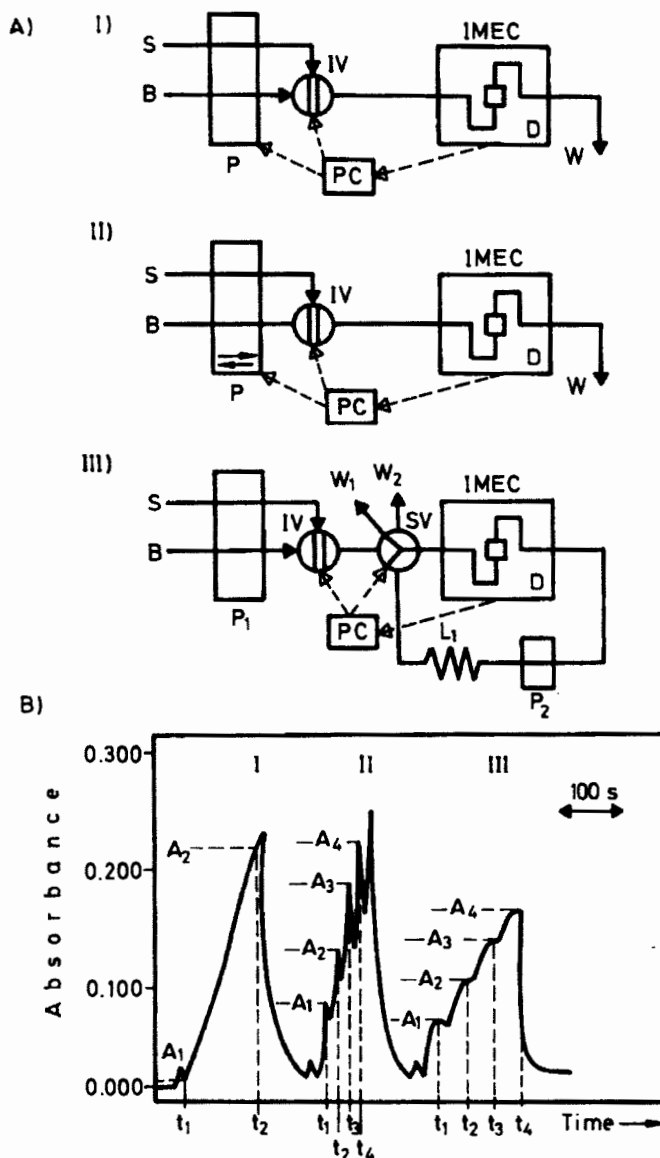
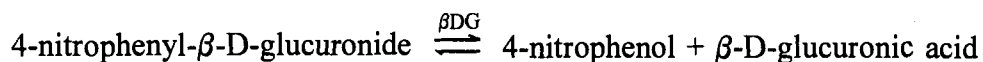


Figure 3.8 — (A) Biosensors used in different FI manifolds to perform reaction-rate measurements: (I) stopped-flow manifold; (II) iterative flow-reversal system; (III) open-closed configuration: S sample; B buffer; P pump; IV injection valve; PC personal computer; IMEC immobilized enzyme cell; D detector; W waste; SV switching valve. (B) Types of recordings obtained by using the three types of biosensors and measurements to be performed on them in order to develop reaction-rate methods. (Reproduced from [50] with permission of Elsevier Science Publishers).

several times through the detector in order to obtain a multi-peak recording such as that shown in Fig. 3.8.B.II. The approach illustrated in Fig. 3.8.A.III involves an open-closed configuration [52] where the injected sample is passed through a switching valve (SV) that is then actuated in order to change from the open to the closed circuit. As a result, the plug is trapped and recirculated along the system with the aid of pump P2, thereby rendering a multi-peak recording (Fig. 3.8.B.III) on repeated passage through the biosensor before SV is switched to send the sample to waste. The recordings provided by both approaches (iterative reversal of the flow direction and use of an open-closed system) enable two types of kinetic measurements, namely: (a) individual reaction-rate measurements based on the absorbance increment between two peaks, whether consecutive or not (or even between minima); and (b) the summation of several individual measurements, which results in improved sensitivity. The performance of these three approaches was critically studied and compared by using the same immobilized β -D-glucuronidase (β DG) biosensor for the determination of glucuronide derivatives based on the following reaction:



The monitored species was the 4-nitrophenol formed in the reaction, which exhibited maximal absorbance at 405 nm.

The comparative study was based on several criteria including the features of the FI approach used and those of the methods developed by using the same biosensor but a different measurement parameter, or by using the same parameter and different FI biosensors.

The complexity of the flow injection manifold required by the three approaches was very similar. All of them necessitated electronic interfaces to control the propulsion and injection systems through the microcomputer in approaches I and II, and the injection and switching valves in manifold III. A passive electronic interface was also required in all three manifolds in order to acquire data from the biosensor/detection system.

The sensitivity of the biosensor based on iterative reversal of the flow direction could be trebled by using the fourth rather than the second peak, to the detriment of sample throughput. The repeatability of the three approaches was excellent (the RSD was less than 2% in every case) [53].

Bioluminescent reactions have been thoroughly investigated with a view to designing integrated flow-through biosensors using firefly and bacterial

luciferase immobilized on various supports; alternatively, immobilization can involve the catalyst for a coupled reaction yielding the hydrogen peroxide for the chemiluminescent reaction, or the luminogenic substrate involved in the light-emission reaction.

The first insoluble derivatives of *bioluminescent enzymes* were prepared by Erlanger *et al.* by reacting luciferases; they investigated the properties of these immobilized enzyme preparations and their potential for studying the mechanism of bioluminescence [54].

Kricka *et al.* reported one of the earliest automated segmented-flow systems using luciferase immobilized on Sepharose CL 6B and packed in a small flow-cell [55]. The biosensor design is depicted in Fig. 3.9.A. The linear range for ATP assays was 3–3000 pmol. The precision of the bioluminescence assay was acceptable (CV = 10.3%), but was significantly boosted (CV = 3.6%) by increasing the wash time to 80 s (carry-over was then 5.9%). Over 100 assays could be performed with the same sensor over a period of 3–4 weeks. The analytical performance of the system was adversely affected by product inhibition as a result of accumulation at the enzyme after repeated use. This drawback was overcome either by prolonged washing (80 s) of the immobilized enzyme or by including Triton X-100 in the wash buffer and using a 20-s wash cycle. The non-ionic detergent acted by promoting removal of the product from the enzyme bed [56]. A more serious problem was posed by microbial growth in the packed bed after prolonged use at room temperature, which significantly raised the background light level, even in the presence of the antibiotic gentamicin.

A segmented-flow system was also used to implement a biosensor based on luciferase immobilized on a nylon coil placed in front of the photomultiplier window of a luminometer [57]. The bioluminescence sensor consisted of a 0.5-m long \times 1 mm ID coil wound around a Plexiglas support. The flow systems used included two lines carrying a luminescent solution and a continuous stream of air into which a 5–80 μ L sample was injected. The determination range was 0.3–100 pmol ATP and up to 20 samples/h could be processed with no carry-over. The RSD for both intra- and inter-assays was 5–8%. Up to 900 ATP samples could be analysed with a single reactor containing *ca.* 0.4 mg of covalently linked enzyme, with a half-life of 15 days. Despite the excellent sensitivity of the biosensor, its proponents, who were aware of the low activity recovery of an enzyme immobilized on nylon tubes, tried other supports for luciferase including epoxy methacrylate beads. Thus, they packed enzyme-loaded beads in a small (3-cm long \times 2

mm ID) glass column that was positioned inside the luminometer. This new sensor was used for the determination of ATP in platelets [58].

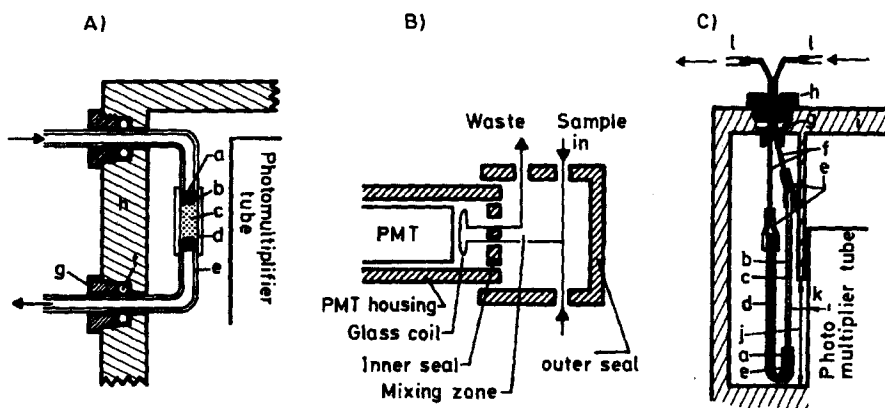


Figure 3.9 — (A) Design of immobilized enzyme-packed flow-cell and its location in the flow-cell adapter: a polyethylene disc; b glass beads; c Sepharose-immobilized enzymes; d Tygon tubing (1/4-in. OD, 3/16-in. ID); e Tygon tubing (3/16-in. OD, 1/8-in. ID); f O-ring; g knurled screw; h flow adapter body. (Reproduced from [55] with permission of the American Chemical Society). (B) Design of the light-tight housing containing the T-piece and the immobilized enzyme coil. (Reproduced from [55] with permission of Elsevier Science Publishers). (C) Design of the enzyme-packed capillary flow-cell: a glass-bead bed support; b capillary tube flow cell; c Sepharose-enzyme bed; d thick-wall capillary for outflow; e silicone rubber tubing; f 22-gauge stainless steel hypodermic needle; g rubber septum; h knurled screw port; i body of sample chamber of LKB 1250 luminometer; j window of sample chamber (38 mm); k distance from flow-cell to PMT (≈ 5 mm); l TeflonTM tubing (50-cm long). (Reproduced from [61] with permission of Academic Press).

Worsfold and Nabi [59] developed a flow-through sensor based on firefly luciferase immobilized on Sepharose 4B or controlled pore glass that was used in the FI manifold shown in Fig. 3.10.A. They used a 6-mm long \times 2.5 mm ID glass coil containing the immobilized enzyme, which was placed in front of an end-window photomultiplier tube. ATP samples and luciferin were simultaneously injected in the merging-zones mode into separate buffered carrier streams by means of a rotary PTFE valve. The detection limit for the Sepharose biosensor was higher by three orders of magnitude and the sensor was more sensitive by two orders of magnitude than was the

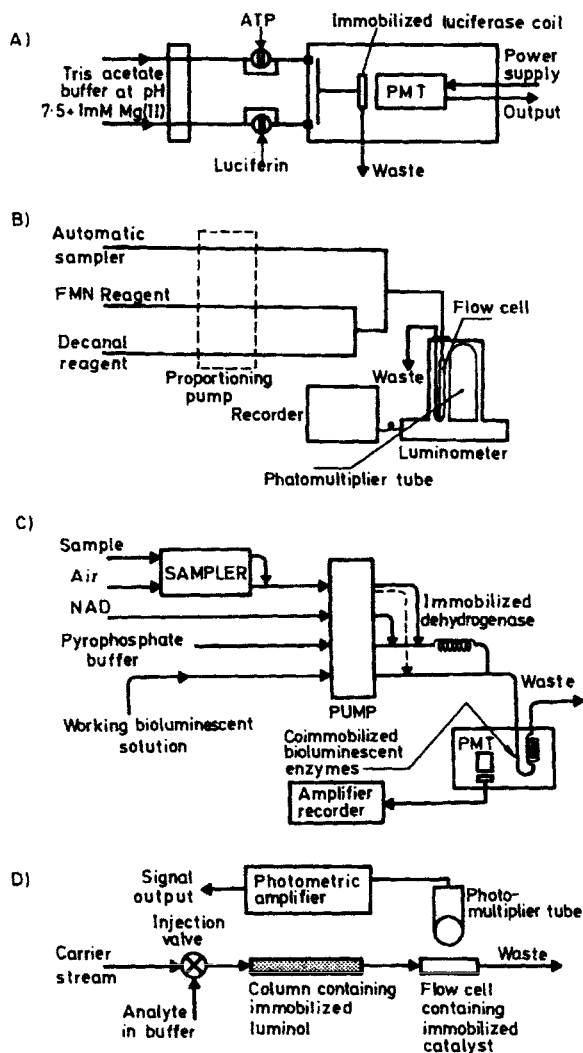


Figure 3.10 — Flow manifolds for implementation of flow-through biosensors. (A) Flow injection merging-zones manifold for the bioluminescence determination of ATP. ATP standards (30 μ L) and luciferin (30 μ L) are injected into the buffered carrier streams, each pumped at 0.7 mL/min and synchronously merged 12.5 cm downstream. Distance from merging point to immobilized enzyme coil, 2.2 cm. (Reproduced from [59] with permission of Elsevier Science Publishers). (B) Completely continuous flow manifold for the determination of NADH. (Reproduced from [71] with permission of the Royal Society of Chemistry). (C) Segmented-flow manifold for the determination of L-(+)-lactate. (Reproduced from [65] with permission of Marcel Dekker, Inc.). (D) Single-channel flow injection manifold with immobilized reagent for the determination of glucose. (Reproduced from [77] with permission of Elsevier Science Publishers).

CPG sensor. The dynamic range for ATP measurements was 1×10^{-12} – 1×10^{-7} M and the RSD was less than 2% throughout that range. While no loss of activity was observed in the Sepharose-bound enzyme when stored at 4°C, its activity was found to gradually degrade on being kept at room temperature in the FI manifold. The manifold used for the ATP assays also enabled creatine kinase (CK) and creatine phosphatase to be assayed. For the enzyme assay, the CK sample was injected into a carrier stream containing ADP and creatine phosphate, and ATP was produced *in situ* as a result. The results obtained for CK standards ranged from 10 to 400 IU/L. Creatine phosphate was quantified with good precision over the range 1×10^{-5} –0.1 M by injecting the sample into a carrier stream containing CK.

Kurkijärvi *et al.* were the first to demonstrate the feasibility of segmented-flow bioluminescence assays by use of a bioreactor packed with bacterial bioluminescent enzymes immobilized on Sepharose 4B [60]. The packed glass column used was placed in front of the photomultiplier tube of a luminometer. The luminescence signal obtained was linearly related to the NADH concentration from 1 pmol to 10 nmol for sample volumes of 2–20 μ L. In the region of 400 NADH assays could be performed with a single enzyme column, with no appreciable change in sensitivity or accuracy. However, problems arising from packing or disruption of the matrix were encountered after 4 days of intensive use.

NADH, glucose-6-phosphate and primary bile acids were assayed by using a flow system identical to that employed for the above-described ATP determination [55]. For this purpose, bacterial luciferase and NADH:FMN oxidoreductase were co-immobilized on Sepharose 4B with either glucose-6-phosphate dehydrogenase or 7 α -hydroxysteroid dehydrogenase, depending on the analyte to be determined. The Sepharose-bound enzymes were packed in small flow-cells that were placed in front of a photomultiplier tube as shown in Fig. 3.9.B. The sensitivity of the co-immobilized bacterial luciferase biosensor was limited by the variable level of background light associated with the NAD–FMN–decanal assay mixture. Measurable concentrations ranged from a few to several hundred pmol. The coefficient of variation was 2–10%, depending on the sample concentration and the duration of the washing step between samples (20 or 80 s). Thirty samples per hour could be assayed and up to 700 consecutive measurements made by using the same biosensor. However, bacterial contamination of the Sepharose limited continuous use of the immobilized preparation. The biosensor design was later modified to reduce background light and improve

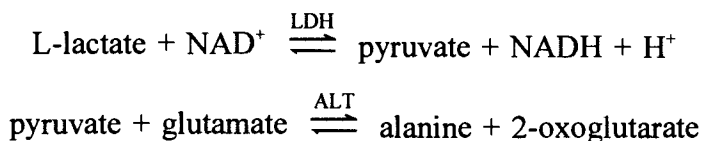
the sensitivity [61]. Thus, both the biosensor bed volume and the reagent flow-rates were reduced, and FMN and decanal were placed in separate, individual reservoirs. These alterations lowered background light to *ca.* 0.1% of its previous level and resulted in a 1000-fold increase in the sensitivity to NADH. The biosensor was constructed from a capillary tube containing Sepharose-bound enzymes (Fig. 3.9.C) and the flow was passed at a rate of 0.09 mL/min through it. The sampling time was 2 min and sampling was followed by a 4-min wash cycle. NADH concentrations from 1×10^{-10} to 1×10^{-6} M in a sample volume of 60 μ L could be measured with an intra-assay CV of 2–10%. The sample concentration range for 6-phosphogluconate achieved by using co-immobilized 6-phosphogluconate dehydrogenase was 1×10^{-9} – 1×10^{-5} M, but contamination of the Sepharose enzyme bed caused the background light emission to gradually increase after only a few hours of operation.

Roda *et al.* developed a luminescence sensor using a segmented-flow configuration for the determination of primary bile acids based on the luminescent enzymes from *V. fischeri*, co-immobilized with 7 α -hydroxydehydrogenase in a nylon coil of 1 m length and 1.0 mm ID [62]. The flow manifolds used included two continuous streams, one containing the working solution and the other carrying air, into which a known volume of sample was intermittently injected. The nylon coil was placed inside the luminometer, in front of the photomultiplier tube window. The assay was highly specific for 7 α -hydroxy bile acid: the standard curve was linear over the range 10–2500 pmol and featured satisfactory precision (CV = 5–10%). By using the same flow manifold and a biosensor containing the bioluminescent enzymes alone, NADH could be measured at concentrations between 1 and 2500 pmol [63] with intra- and inter-assay precision of 5–10% at a rate of over 20 samples/h with no carry-over. Ethanol, glycerol, aldehydes, and 3 α -, 7 α - and 12 α -hydroxy bile acids were also determined by using bioluminescent enzymes and suitable dehydrogenases immobilized on separate nylon coils [64]. The dehydrogenase reactors were placed on the outside of the luciferase–oxidoreductase flow-cell, thereby conforming the biosensor as shown in Fig. 3.10.B. The segmented-flow system used comprised four streams: the first contained the working bioluminescent solution, the second and third supplied the immobilized dehydrogenase with NAD solution and sodium pyrophosphate buffer of pH 9, and the fourth was air into which samples were injected. Picomolar amounts of analytes could thus be determined with acceptable repeatability. A bioluminescence segmented-flow

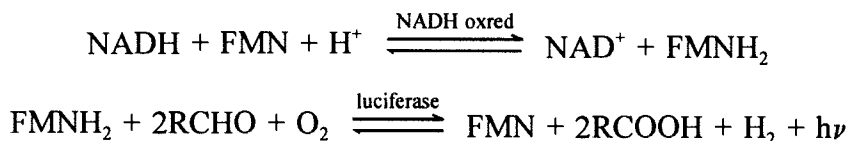
sensor for the determination of L-alanine in serum was later developed that used an alanine dehydrogenase reactor detached from the biosensor [65]. Urine samples had to be deproteinated and diluted prior to use, while serum samples only required dilution following filtration. The bioluminescence signal was linear from 50 to 1500 pmol and 25–30 measurements per hour could be made. The residual activity of the overall enzyme system was *ca.* 50% of its initial value after 2 months of use at a rate of 50 samples/h. The enzyme coils were employed at room temperature and stored at 4°C. By using a similar biosensor with leucine dehydrogenase in the reactor preceding the biosensor, branched-chain L-amino acids were determined at concentrations of 20–2000 pmol in serum and urine [56]. The catalytic activity of serum L-lactate dehydrogenase was measured by monitoring the NADH produced by the enzyme with the bioluminescent flow system using nylon-immobilized bacterial enzymes [67]. The dehydrogenase-catalysed reaction between L-lactate and NAD took place in a flow-through mixing coil preceding the biosensor. The response was linear over the ranges 1–5000 and 3–2000 IU/L at 37 and 25°C, respectively.

Nabi and Worsfold [68] used a flow injection manifold coupled to a biosensor for the determination of NADH, NADPH, FMN and FMNH₂. They employed a partially purified extract from *V. harveyi* containing bacterial luciferase and oxidoreductase to immobilized cyanogen bromide on activated Sepharose 4B. The bound enzyme was placed in a glass coil in front of the photomultiplier tube as shown in Fig. 3.9.B. For NADPH measurements, the sample and decanal were simultaneously injected into separate carrier streams both containing FMN and dithiothreitol (DTT) (Fig. 3.10.B). Flavin mononucleotide (FMN) was determined by simultaneously injecting it and NADH into separate carrier streams containing decanal and DTT. On the other hand, FMNH₂ was determined by using two streams containing DTT alone and injecting the analyte and decanal into each. The calibration graph for the bioluminescence determination was linear from 1×10^{-9} to 1×10^{-4} M, with an RSD of 1.2–6.6%. The enzyme activity was preserved for several days at room temperature within the FI manifold, but the purity of the extracted enzymes was a major factor in determining the biosensor's limit of detection. For ethanol assays, alcohol dehydrogenase was immobilized on the cyanogen bromide-activated Sepharose 4B support and inserted upstream of the biosensor. The detection limit thus achieved was 1×10^{-6} M and the RSD was 1.8% ($n = 5$) for 1×10^{-6} –0.1 M ethanol. Alcohol dehydrogenase could also be assayed in the range 0.03–30 pmol.

More recently, Girotti *et al.* [71] reported a bioluminescence flow-through sensor for the determination of L-(+)-lactate using on co-immobilized lactate dehydrogenase and alanine aminotransferase (ALT), and, separately, immobilized bacterial luciferase + NADH oxidoreductase, all in nylon coils that were inserted in a flow manifold such as that shown in Fig. 3.10.C. A volume of 20 μL of sample was injected into an air stream that was subsequently merged with a mixture of co-enzyme and buffer, and passed through the LDH+ALT enzyme reactor, where the following reactions took place:



The mixture was finally merged with the bioluminescent solution (FMN + DTT) prior to arrival at the biosensor (a 5-mm \times 0.5 mm ID nylon coil containing the luminescent enzymes wound around a Plexiglass support positioned in front of the photomultiplier tube), where the following reactions occurred:



The LDH+ALT reactor provided a linear response from 0.1 to 50 $\mu\text{mol/L}$ lactate, thereby increasing lactate conversion by 117–183% relative to LDH alone. The intra- and inter-assay CV were both less than 5%, and recoveries ranged from 93 to 106%. Even though roughly 100% of the LDH and ALT added bound to the support under the immobilization conditions used, the activities of the immobilized enzymes were *ca.* 3% of those of the free enzymes, which is consistent with previous results obtained by the same [67] and other authors [69,70]. Jointly immobilized LDH and ALT preserved *ca.* 50% of their original activity after 60–90 days of intermittent use. On the other hand, immobilized luciferase was less markedly inhibited than that in the free solution by substances present in the biological samples assayed [71].

At a later stage, Girotti *et al.* developed a highly sensitive and rapid bioluminescence flow-through sensor for the determination of the L-phenylalanine (Phe) in serum by monitoring the reduced form of nicotinamide

adenine dinucleotide produced by immobilized phenylalanine dehydrogenase (PHeDH) with bacterial bioluminescent enzymes immobilized in a separate nylon coil. They investigated three PHeDHs extracted from different sources and analysed their performance. The *Bacillus radius* reactor used was found to result in the highest conversion rate and sensitivity; also, the response was linear from 1 to 100 μM at 25°C, and the detection limit was 10 pmol (0.5 μM). The intra- and inter-assay CV were both less than 5% and recoveries ranged from 90 to 101%. The results obtained by using this biosensor in conjunction with the above-described manifold were quite consistent with those provided by a chromatographic method for the determination of Phe in serum, as well as with normal reference values [72].

There are several available integrated *flow-through biosensors based on chemiluminescence detection* using hydrogen peroxide and —usually— luminol. In this context, there are some antecedents of Nieman's work in the experiments of Murachi *et al.* [73] aimed at the determination of glucose and lactic acid in serum by use of a conventional FI system and immobilized enzymes, and those of Ruzicka and Hansen [74] in relation to the determination of glucose, creatinine, free cholesterol and lactic acid by means of specific oxidases as H_2O_2 -producing enzymes immobilized on CPG and inserted in a FI manifold including a chemiluminescence detector based on the luminol/ferricyanide system.

With a view to facilitating use of the chemiluminescence of luminol for analytical purposes, Nieman developed several approaches to preparing the required reagents in immobilized or solid-state form. He employed FI manifolds to test the proposed approaches. In the beginning, he used the reagent in immobilized form to pack a column placed in an FI manifold preceding the detector. Luminol was covalently attached to the support particles via various silane and linkage molecules. Loadings of 500–100 μmol luminol per gram of support were thus achieved by covalent binding and up to 30 $\mu\text{mol/g}$ by adsorption. At a later stage, Nieman used the immobilized catalyst for the determination of hydrogen peroxide (an analyte that can be enzymatically converted into hydrogen peroxide) of analytes labelled with luminol or a related compound. One of the biosensor approaches he tested involved using peroxidase, an efficient catalyst for luminol CL, placed in the observation cell. A combination of the previous two approaches led to a flow-through biosensor where luminol was used in immobilized form in a packed reactor included in an FI manifold, and covalently immobilized peroxidase was placed in the biosensor flow-cell

[75]. Niemann and Hool tested alternative immobilized species including hemin and hemoglobin [76].

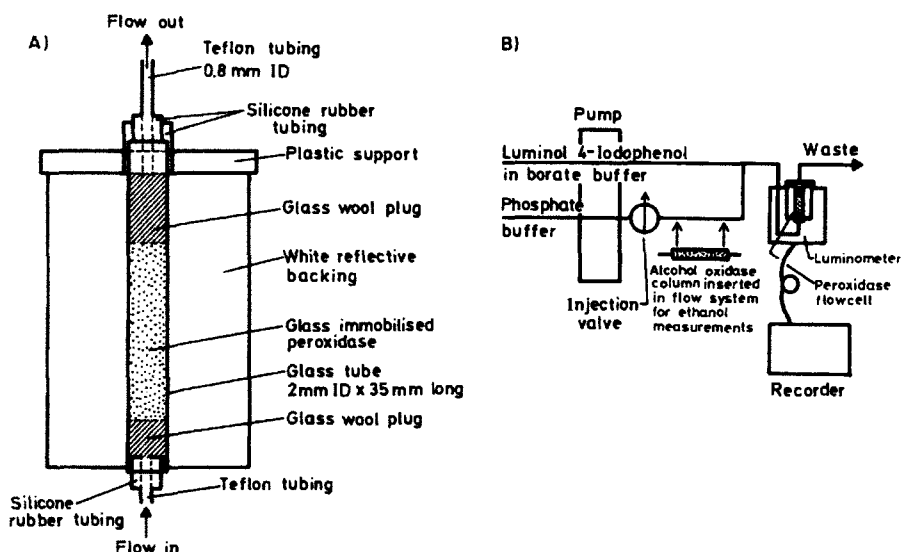


Figure 3.11 — (A) Immobilized peroxidase sensor. Glass-immobilized peroxidase is packed in the flow-cell shown. The plastic support plate fits the top surface of the photomultiplier chamber of the immunometer so as to support the vertically held flow-cell in front of the photomultiplier itself. (B) Flow system for hydrogen peroxide/ethanol determinations. For ethanol determinations, the immobilized alcohol oxidase column is inserted immediately after the injection valve (shown by the arrows). Luminol ($62\ \mu\text{M}$) and 4-iodophenol ($0.4\ \mu\text{M}$) are dissolved in 200 mM borate buffer (pH 8.9) and pumped at a flow-rate of 0.8 mL/min. Phosphate buffer (10 mM, pH 7.0) is pumped at 1.6 mL/min. (Reproduced from [78] with permission of Elsevier Science Publishers).

Subsequently, Nieman *et al.* carried out an exhaustive study of various solid supports and immobilization procedures for use in biosensors containing luminol deposited on particles used as the packing for flow-through columns used in the determination of hydrogen peroxide. They assayed covalent attachment via glutaraldehyde and an alykylsilane on silica and Ambersorb, adsorption on Ambersorb, and anion exchange on a resin. The amounts of bound luminol obtained by adsorption on Ambersorb, covalent attachment and ion exchange were 29, 82 and 875 $\mu\text{mol/g}$ support, respectively. Such amounts were more than adequate for hundreds of deter-

minations with a single column. Covalently bound or adsorption-immobilized luminol was released into the flow stream by injection of an alkaline sample. The injected solution functioned to control the pH of the channel including the Ambersorb, but silica consumed hydroxide and altered the pH, thereby degrading analytical performance. On the other hand, the anionic concentration of the injected sample determined the amount of luminol released and the pH downstream the column when the luminol was immobilized by ion exchange. The resulting hydrogen peroxide detection limits were 0.5, 1–2 and 5–10 μM for adsorption, covalent binding and ion-exchange immobilization. The near-neutral pH used with the ion-exchange immobilization was compatible the enzyme reactions involved. Glucose was determined by using a single-channel FI manifold such as that shown in Fig. 3.10.D, immobilized luminol and a biosensor based on immobilized horseradish peroxidase and glucose oxidase. The high, variable ionic strengths involved in the ion-exchange immobilization procedure required application of an ion-replacement sample pretreatment method [77].

Figure 3.11 shows a flow-through bioluminescence sensor and the integrated FI configuration used in conjunction with it for the determination of ethanol. The sensor consisted of peroxidase immobilized on glass beads that were packed in a piece of glass tubing (35 mm \times 2 mm ID \times 0.5 mm wall thickness). One end of the column was sealed with a plug of glass wool and the other was connected to PTFE tubing via pieces of narrow-bore silicone rubber tubing. The overall length of the peroxidase–glass column was 15–20 mm. The whole column was inserted into a Perspex top-plate that held a white reflective surface. This was in turn placed in the luminometer sample chamber and the column was connected to the flow manifold. A volume of 100 μL of sample was injected into a phosphate buffer of pH 7.0 for optimal development of the oxidation of ethanol in an alcohol oxidase reactor. Subsequently, the plug was merged with a luminol stream and passed upstream through the column. The analyte could thus be determined at concentrations as low as 1×10^{-9} M (1 pmol), with a linear response down to 1×10^{-6} M. Continuous use of the immobilized peroxidase sensor at room temperature for 3–4 months resulted in a signal decrease of only 15–20%, which testifies to the high overall stability of the enzyme [78].

The chemiluminescent reaction between *bis*(2,4,6-trichlorophenyl)oxalate (TCPO) and hydrogen peroxide in the presence of perylene was used by Seitz *et al.* to develop what is seemingly a prototype for the determination of H_2O_2 based on glucose oxidase immobilized on controlled pore glass

(CPG). The experimental set-up used for this purpose consisted of an infusion pump that propelled three syringes containing TCPO and perylene in ethyl acetate, methanol, and an aqueous buffer (ethyl acetate was the most suitable solvent for TCPO and methanol was needed to render the solvent water-miscible). The ethyl acetate and methanol streams were merged prior to the biosensor. On the other hand, the buffer flowed through the sampling valve, then through the enzyme column and finally into the CL cell. The sample was introduced into the system by drawing it into a 1-ml loop and switching the sampling valve. The sample was thus pushed by the buffer out of the loop and into the CL cell. A column packed with glucose oxidase immobilized on CPG was inserted between the valve and the CL cell in the flow manifold for the determination of glucose. Mixing in the cell was aided by N₂ bubbling, and any peroxide in the sample reacted with TCPO to yield CL. The light from the CL cell was detected by a photomultiplier, amplified and recorded. In this way, glucose was determined in urine with no interference from uric acid [79].

3.2.1.2 Flow-through electrochemical biosensors

The essential difference between the biosensors described in Section 3.2.1.1 and those dealt with in this Section is that, while the former must have a minimal flow-cell void volume in order to provide adequate sensitivity because measurements are made on the solution held in the cell, electro-analytical sensors rely on measurements during the process that takes place at the electrode/solution interface —the solution must be in contact with the sensing surface, so the cell volume is not a limiting factor.

Electrochemical flow-through sensor development can benefit from the extensive experience gathered in studying and using batch biosensor measurements. However, two major features of flow experiments should always be borne in mind, namely: (a) the active surface of the sensor must be mechanically strong enough to withstand liquid flow; and (b) except for biosensors used in continuous monitoring, the analyte forms a zone of a given shape and width in the stream, and its residence time in the analytical system is not very long. As a result, the time constants of all the processes occurring between sample introduction into the stream and display of the analytical signal must be small enough for distortion of the zone profile and the signal to be suppressed. In addition, the very small cells used in liquid chromatography exhibit a high impedance and a behaviour that deviates from the general theory, which relies on the assumption that the cell dimensions

are much larger than the thickness of the diffusion layer. Consequently, the detection conditions should always be derived for measurements performed in the given flow-cell rather than from data obtained with large cells in batch experiments.

3.2.1.2.1 Amperometric flow-through biosensors

Amperometric biosensor development is still a steadily growing area of electrochemistry. Improvements in the stability, selectivity and scope of amperometric biosensors remain highly desirable achievements in order to meet new challenges posed by clinical and environmental samples. The usefulness of these biosensors is often hampered by gradual fouling of the surface through adsorption of large organic surfactants or reaction products, which, in addition to producing parasitic signals, may severely affect the catalyst integrity. Amperometric sensors lack the ability to discriminate between solutes with similar redox properties. Also, sensing of a number of major solutes is often hindered by their slow electron transfer kinetics at the commonly used electrode materials.

A biological compound (an enzyme, usually) intended to improve the response of an electrode can be incorporated into it in two ways, namely: (a) by altering the sensing surface in order to accommodate the biocatalysts [*i.e.* by constructing a (bio)chemically modified electrode]; and (b) by using a membrane placed in front of the surface electrode in order to trap the enzyme. The enzyme can be used in isolation (most often in a commercially available form) or be part of a tissue material or bacterial cells.

The description below provides an overview of the wide variety of existing amperometric biosensors based on the most representative examples of both straightforward and more sophisticated, special designs, and micro-electrodes using previously isolated enzymes, which are dealt with in this order. Finally, a description is made of sensors using a biological (animal or vegetable) tissue or microorganisms.

Most of the amperometric flow-through biosensors based on commercially available enzymes are employed to measure consumed or released oxygen by using a Clark electrode or a solid-state electrode to monitor the hydrogen peroxide formed or an enzymatically reduced acceptor.

Glucose oxidase, one of the most widely used enzymes for constructing biosensors, was employed by Matuscewski and Trojanowicz to develop a biosensor based on a mixture of graphite powder, silicone oil and the biocatalyst. The paste thus obtained was placed in a slot of the PTFE end

of the electrode body. The device exhibited a linear response in a single-line manifold at glucose concentrations of up to 30 mM and allowed up to 120 samples/h to be processed. The detection limit for a 750- μ L injected sample volume was 20 μ M glucose. The average lifetime of the biosensor was 3 weeks, with no significant decrease in sensitivity. The analytical potential of this probe was assessed on the determination of glucose in fruit juices and carbonated soft drinks [80].

Chi and Dong exploited the electrocatalytic oxidation of the hydrogen peroxide produced by a palladium-modified glassy carbon electrode (GCE) to develop an amperometric sensor for glucose constructed by electrochemical co-deposition of palladium and glucose oxidase at -0.9 V for 15 min. The resulting modified surface was coated with a thin film of Nafion. The sensor was successfully employed in the determination of glucose over the concentration range from 0.001 to 8 mM, with no interference from such species as ascorbate or saccharides [81].

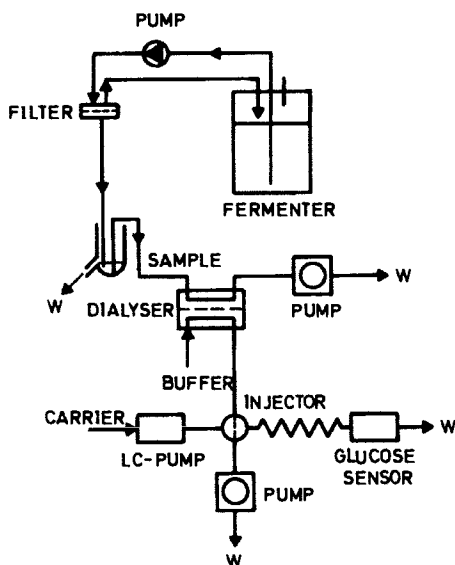


Figure 3.12 — Interfacing of a fermenter to an FI system. The fermenter medium is continuously recycled by a pump to the filter unit, from which the filtrate is guided to a small reservoir (500 μ L). The sample solution is aspirated through a dialyser, the acceptor stream of which is fed to the injector of the FIA system. The analyte content is assayed amperometrically by using the glucose sensor incorporating the enzyme-containing chemically modified electrode. (Reproduced from [86] with permission of Elsevier Science Publishers).

Many biosensors rely on mediators to act in the electrode step of the detection process. Such mediators are used both as film coats over the sensor and as carrier solutions—which obviously results in more complicated and expensive determinations. Biosensor mediators are usually organic compounds [82,83] or, to a lesser extent, inorganic substances [84], depending on the particular enzyme used and species to be determined.

Smyth *et al.* [85] developed a bioamperometric sensor in which the enzyme (glucose oxidase) was co-immobilized with the redox polymer $[\text{Os}(\text{bpy})_2(\text{PVP})_{10}\text{Cl}]\text{Cl}$ (where bpy and PVP denote bipyridyl and poly-4-vinylpyridine, respectively) and glutaraldehyde on the surface of a platinum electrode, and subsequently coated with an electropolymerized layer of pyrrole also containing glucose. The electron transfer from the reduced flavine adenosine dinucleotide (FADH_2) group in the enzyme core to the electrode surface was facilitated by the redox polymer/pyrrole system. The high permeability of both layers allowed the direct amperometric detection of β -D-glucose over the linear concentration range 1–50 mM.

An inexpensive, easy-to-construct, disposable biosensor based on a chemically modified graphite electrode including an adsorbed cross-linked layer of glucose dehydrogenase and the use of mediators was employed in conjunction with a flow injection manifold for on-line monitoring of glucose in fermenters. The electrode was constructed from graphite rods of 3.1 mm diameter, and was polished, washed, dried at 60°C for 3 min and heated in a muffle furnace at 600°C for 90 s to remove small graphite particles from the surface. It was then modified by applying a diethyl ether solution of a Nile Blue derivative of terephthalic acid onto the end surface. After all the ether had evaporated (within a few minutes), the electrode was washed with de-ionized water and tested by cyclic voltammetry. The surface coverage was estimated from the area of the oxidation peak and the electrode was discarded if the peak was too low or broad. A coverage of *ca.* 5–6 nmol/cm² was typically found to be optimal. The enzyme was immobilized onto the electrode by applying a mixture consisting of 5 μL of enzyme solution and 5 μL of glutaraldehyde for about 10 min. The electrode was finally washed with phosphate buffer to remove excess mixture. The biosensor was investigated for serviceability and used to determine glucose in a wine fermentation tank by using an FI system such as that shown in Fig. 3.12; potential contamination of the sensor was avoided by using a filter and including a dialysis step, which ensured proper operation for a least 3 days of continuous use [86].

The stability and durability of chemically modified electrodes can be improved by coating them with an appropriate membrane. Such is the case with the sensor reported by Gorton *et al.*, constructed from carbon paste modified with glucose dehydrogenase, nicotinamide adenine dinucleotide and a mediator (Meldola Blue). The biosensor surface was protected by a membrane of poly(ester sulfonic acid) cation exchanger that prevented aqueous soluble species from dissolving out the biosensor. Its performance was evaluated by using it in a flow injection system, where it provided calibration curves that were linear between 100 μM and 20 mM glucose at +100 mV *versus* Ag/AgCl, and a throughput of 40 samples/h. The biosensor response remained stable for at least 2 weeks.

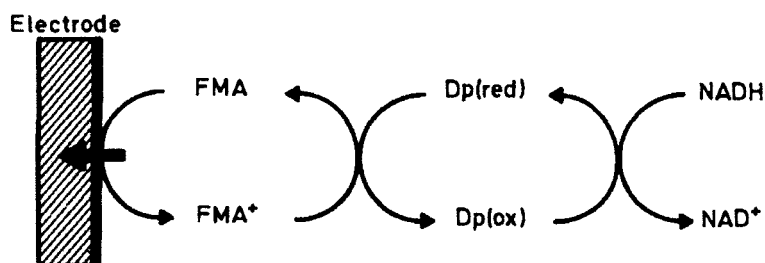


Figure 3.13 — Set-up for the electrocatalytic oxidation of NADH catalysed by the reduced form of diaphorase (Dp). Dp (red) and Dp (ox): reduced and oxidized form of diaphorase. (Reproduced from [88] with permission of the Royal Society of Chemistry).

Dissolved ferrocenylmethanol was used as mediator in several kinetic studies of NADH-dependent dehydrogenases involving a glassy carbon electrode onto which the enzyme diaphorase (Dp) was immobilized by cross-linking with glutaraldehyde. The sensor consisted of a glassy carbon disc mounted in a polytetrafluoroethylene rod the surface of which was polished with alumina. The immobilized Dp electrode was prepared by mixing the enzyme with glutaraldehyde at the electrode surface, which was then kept at room temperature for 2 h in order to allow surface polymerization. A scanning electron microscopic measurement revealed the thickness of the enzyme film to be *ca.* 0.01 mm. The biosensor exhibited outstanding stability and the current response reached a steady state within 2–3 s on addition of NADH, which reacted according to the sequence shown in Fig. 3.13. As

regards practical applications, the biosensor was used for the determination of lactate dehydrogenase [88].

A C felt sensor coated with a glucose oxidase monolayer was used in an FI manifold in the presence of dissolved potassium hexacyanoferrate as mediator for the on-line determination of interferents [89].

The fast response and long-term durability of GOD/pyrrole electrodes make them suitable for use as flow-through biosensors. Because they oxidize enzymatically produced hydrogen peroxide, the working potential normally has to be adjusted to 600 mV *vs* SCE, which results in co-oxidation of interferents present in the analyte solution and necessitates compensation. Conducting organic polymer films such as pyrrole, polyaniline and polyphenol were recently shown to exhibit a size-exclusion selectivity that depends on the polymerization conditions and, especially, the thickness of the polymer film [90,91]. This knowledge has allowed the plateau current for ascorbic acid to be lowered by a factor of 10 using polypyrrole electrodes obtained by a total transferred charge of 6.5 mC/mm² during the electrochemical polymerization reaction instead of a bare platinum electrode surface. Thicker polymer films might allow the response to ascorbic acid at 600 mV *versus* SCE to be even further lowered; however, such films have an adverse effect on the response characteristics of the sensor for enzymatically produced H₂O₂. Based on these observations, a flow injection system for the selective determination of glucose in the presence of interferents was constructed. A four-electrode flow-through electrochemical cell furnished with a GOD/polypyrrole and a serum albumin/polypyrrole electrode was designed for that purpose and used in an automatic FI system [92] as shown in Fig. 3.14.A. The major facts to focus on were the mass transport characteristics through the polymer layer at the enzyme electrode and the albumin electrode. On the assumption that the thickness of the polypyrrole films are equal, both electrodes should possess analogous properties as regards substrate diffusion, the amount of hydrogen peroxide formed and interferents present at the electrode surface. In fact, the peak height and signal rise time for both electrodes were found to be comparable for ascorbic acid and hydrogen peroxide, whereas glucose was only determined by the GOD-modified electrode. The overall system was tested on various natural samples including fruit juices and wines with excellent results.

A combination of a biosensor assembly such as that described in the previous paragraph and several enzyme reactors allows simultaneous

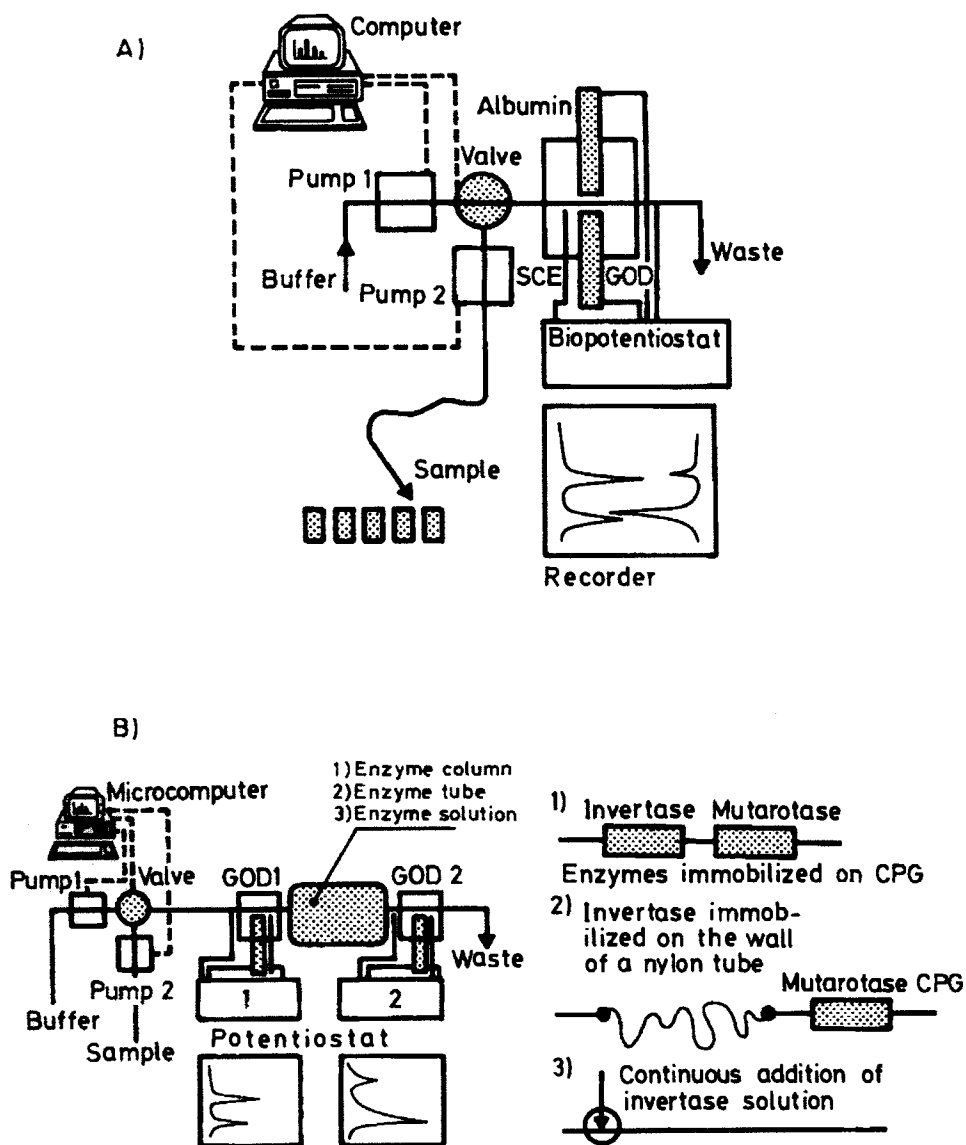


Figure 3.14 — (A) Flow injection system for the determination of glucose in the presence of interfering compounds. (Reproduced from [92] with permission of Elsevier Science Publishers). (B) Flow injection manifold for the simultaneous determination of sucrose and glucose. (Reproduced from [93] with permission of the American Chemical Society).

determinations to be accomplished with no interference from glucose, which is of interest for food analysis and fermentation monitoring [93–95]. Thus, sucrose and glucose were determined simultaneously by using a combination of two GOD/polypyrrole sensors and immobilized invertase and mutarotase, in conjunction with the FI manifold depicted in Fig. 3.14.B. Glucose was determined with the first GOD/polypyrrole sensor and sucrose was then hydrolysed to fructose and β -D-glucose in a reaction catalysed by invertase, which was either immobilized on CPG and packed in a column or immobilized directly onto the walls of a nylon tube. An enzyme column containing mutarotase immobilized on CPG was used to accelerate equilibration between the glucose anomers. Total glucose was then measured with the second GOD/polypyrrole biosensor. The disaccharide cleavage was found to seemingly depend on the immobilization technique used for invertase: immobilization onto the inner walls of a nylon tube led to more efficient hydrolysis of the disaccharide than did immobilization on CPG [96].

One other way of increasing the selectivity of a flow-through sensor is by coupling it on-line with a separation technique suited to the characteristics of the sample matrix and sampling conditions. A coupled configuration of this type was used by Mascini *et al.* for *in vivo* monitoring of glucose. The overall system consisted of a subcutaneous microdialysis probe coupled to a glucose biosensor accommodated in a thin-layer flow-cell [97]. The biosensor was constructed by placing a nylon net (100 μm thick \times 6 mm diameter) loaded with immobilized glucose oxidase over the electrode (a platinum disk 3 mm in diameter). A thin (20 μm) membrane of cellulose acetate was stretched over the entire plastic block where the electrode area was located in order to remove electrochemical interferents such as uric acid and ascorbate, which were prevented from reaching the electrode surface while hydrogen peroxide was allowed to pass through easily. The operational principle of the system was as follows: a physiological buffer was pumped at a constant flow-rate of 10–30 $\mu\text{L}/\text{min}$ into a thin dialysis fibre of 200 μm diameter (a microdialysis probe) that was placed subcutaneously or into the bloodstream; the buffer equilibrated with the subcutaneous fluid or the blood and then flowed into a thin-layer cell furnished with the glucose biosensor for continuous monitoring of the glucose value. The performance of a commercially available probe and a single sterilized thin dialysis hollow fibre as microdialysis probe was compared. The approach offers some advantages over insertion of a bioprobe needle into the body; thus, the biosensor sensitivity can be checked regularly, the hollow fibre used can be readily

sterilized and inserted into animals and humans while awake, the overall *in vivo* implantation process can be controlled much more strictly, and such events as inflammation and clogging (on insertion into the bloodstream) can readily be followed and prevented.

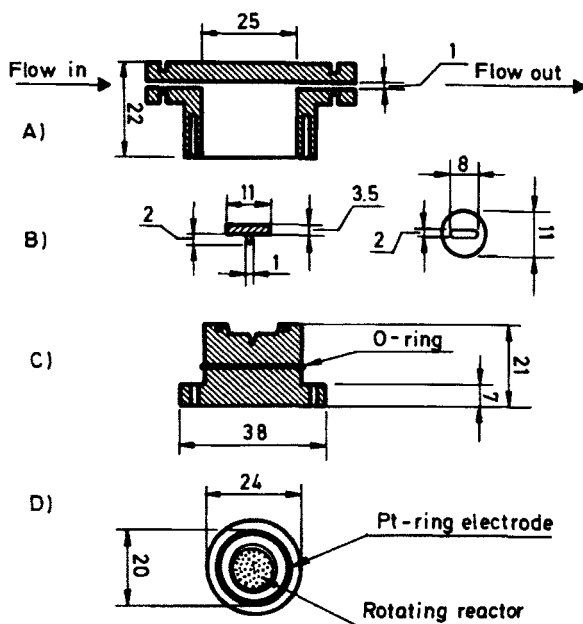


Figure 3.15 — Elements of flow-through cell containing a rotating reactor and platinum ring electrode. (A) Upper cell body defining the flow-through reaction/detection chamber. (B) Rotating reactor showing positioning needle point. (C) Lower cell body showing the well for the rotating reactor and the concentric Pt-ring electrode. (D) Top view of the lower cell body. All dimensions are given in millimeters. The lower cell body is screwed to the upper cell body for cell assembling. (Reproduced from [98] with permission of the American Chemical Society).

Recently, Mottola [98] reported a sensor based on the disk-ring principle previously developed by Kamin and Wilson [99], and Wang and Lin [100]. Unlike Mottola's design, its forerunners involved no stationary ring electrode or rotation of the reactor part; in addition, their reactor/electrode was located at the cell bottom. In Mottola's assembly, a product of an enzyme-catalysed reaction at a bioreactor rotated at a constant speed was hydrodynamically transported to a stationary ring electrode, where it was electrochemically monitored. The sample was transported to the detection unit by an un-

segmented continuous-flow stream. The design, depicted in Fig. 3.15, circumvents problems associated with shaft rotation, which are difficult to adapt to continuous-flow sample/reagent processing, and takes advantage of the good hydrodynamic environment in the vicinity of the ring electrode. This approach provides increased sensitivity by use of a very small amount of immobilized biocatalyst. Mottola used it to measure the hydrogen peroxide produced in the glucose oxidase-catalysed oxidation of glucose by dissolved oxygen with the aid of a platinum electrode. The enzyme was immobilized on CPG, which can readily be deposited uniformly on one side of double-coated sticking tape. The tape was then affixed to a disc that was spun. Rotation of the "enzyme reactor" minimized or even overcame the problem associated with deceleration of reactions in low-dimensional spaces. This phenomenon is characteristic of immobilized reagents confined in one-dimensional spaces such as those of pores and other low-dimensional fractal surfaces, and reflects in the fact that the Michaelis–Menten constant for immobilized enzymes is usually somewhat higher (the initial reaction rate is lower) than for the same enzyme in solution as a result of diffusional constraints. These shortcomings can be minimized by decreasing the particle size of the inner support or, as actually done by Mottola, by efficient stirring. Due care should be exercised, however, to avoid hindering free flow in packed reactors as a result of too small a support particle size.

Batch injection analysis (BIA) is a recent methodology involving injection of a sample from a micropipette tip towards a nearby detector immersed in a large volume of a stirred solution held in a batch wall-jet cell in order to reduce both the sample volume and reagent consumption [101]. Owing to the difficulty involved in handling solutions in BIA, use of a highly selective detector is virtually mandatory. In this respect, enzyme electrodes may be regarded as appropriate probes for obtaining selective, sensitive measurements in BIA. Amine and Kauffmann developed several biosensors for use in BIA by casting immobilized glucose oxidase (GOx) and xanthine oxidase (XOD) on an electrode surface. The analyte is quantified by amperometric detection of the product of the enzyme reaction, *viz.* hydrogen peroxide, at +650 mV vs Ag/AgCl (GOx), or the reduced form of Methylene Blue at +50 mV vs Ag/AgCl (XOD). One other sensor developed by these authors consists of glutamate dehydrogenase incorporated into a carbon paste electrode used for direct measurements or detection of the product of the phenazine methosulphate-mediated oxidation at the enzyme electrode. The cell cover has four holes that can be switched from the BIA to the FIA mode

as shown in Fig. 3.16. In the BIA mode, one hole is located exactly opposite the surface disc of the working electrode and accommodates a standard pipette. The tip of the pipette is fixed at a preset distance from the electrode surface that may be adjusted by moving the electrode up or down via a PVC adapter seal. Two other holes are used to hold the counter and reference electrodes in place. The inlet and outlet allow the solution to be drained without removing the electrode. The fourth hole is used for sample and reagent addition in the steady-state mode. In addition to increased equipment simplicity, higher throughput and sparing carrier consumption, the use of BIA in conjunction with a mediator surpasses FIA in the fact that the reduced form of the mediator, which is not oxidized at the electrode, is readily oxidized by dissolved oxygen. This allows the mediator to be recycled, in contrast with FIA, where it is wasted. In addition, any risk of memory effects is eliminated thanks to the gradual increase in the reduced mediator concentration on repeated injections [102].

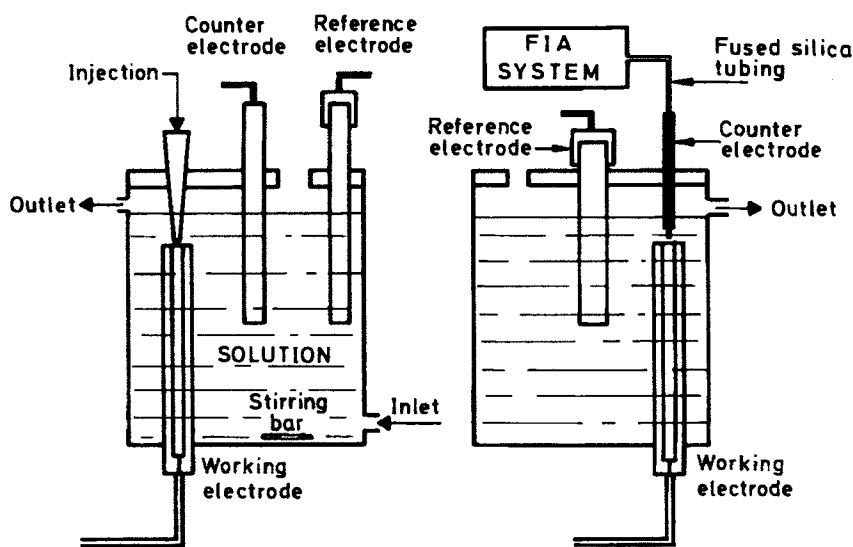


Figure 3.16 — Schematic diagram of cell designs for BIA (left) and FIA (right). (Reproduced from [102] with permission of Elsevier Science Publishers).

Groom and Luong [103] designed a flow-through biosensor for cyanide consisting of a column packed with rhodanase immobilized on a pre-activated nylon membrane attached to the surface of a hydrogen peroxide

electrode. Immobilized rhodanase converted cyanide to thiocyanate ion in the presence of thiosulphate ion, and the sulphite formed was in turn converted into sulphate in the presence of sulphite oxidase. The hydrogen peroxide produced was determined electrochemically at a platinum electrode using Ag/0.7 M AgCl as reference. The calibration graph was linear from 5 to 1000 μM cyanide. The results compared well with those obtained in dissolved enzyme and colorimetric assays, but not with those provided by the commercially available Orion cyanide electrode. The biosensor was successfully applied to the determination of cyanide in brass plating and rinsing solutions.

Miniaturization as a general trend in science and technology has also reached flow-through sensors as regards both design and construction of the sensor proper and the experimental set-up or flow manifold used prior to detection.

One immediate approach to sensor miniaturization involves reducing size. Such is the case with the design reported by Ikariyama *et al.*, who constructed micro-platinum electrodes that were 10–500 μm in diameter from the acrylic block of a thin-layer cell. Following reductive deposition of platinum particles on a platinum wire, glucose oxidase was bound to the porous metal surface via glutaraldehyde. The electrode exhibited a high sensitivity (the lowest detection limit was 0.5 μM) and a wide dynamic range (0.5 μM –20 mM) in batch operation that decreased for flow measurements (lowest detection limit 50 μM) through dispersion of the sample in the flow manifold or a poor signal-to-noise ratio derived from a peristaltic wave produced by the double-plunger pump used. These authors made a variety of enzyme electrodes in diameters from 10 μm to 3 mm in order to analyse the relationship between size and the measuring performance of the associated detection system. An enzyme electrode of 3000 μm diameter was used as reference to determine the effect of miniaturization. The flow-rate employed was 0.8 mL/min. Figure 3.17 illustrates the relationship between the enzyme electrode diameter and the time needed to measure each glucose sample. The solid line shows the time when each sample peak returned completely to the baseline and the dotted line indicates the time during which no interaction between sample peaks was observed on continuous injection of glucose sample. Obviously, the smaller the enzyme electrode used was, the faster measurements were carried out. One of the greatest assets of microfabricated enzyme electrodes is the high sample throughput they provide as a result of being in shorter contact with injected samples.

The micro-enzyme electrode of 10 μm diameter allowed up to 600 glucose samples per hour to be measured and provided a stable response after 4 weeks and 1000 glucose injections [104].

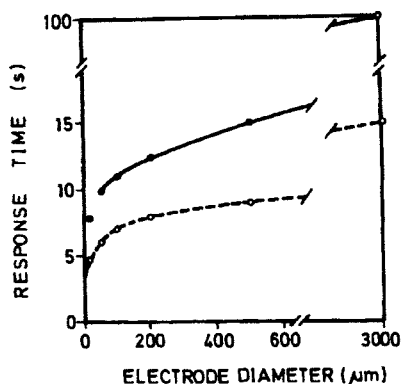
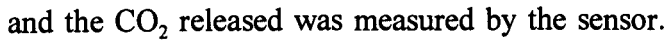


Figure 3.17 — Relationship between the micro-enzyme electrode diameter and its response time. The response times for single injections and repeated injections are represented by a solid and dotted lines, respectively.

Karube *et al.* designed two types of micromachined biosensors: one was based on a micro Clark-type oxygen electrode [105] made by photolithography and anisotropic etching [106], and was constructed in disposable versions for carbon dioxide [107], L-lysine [108] and hypoxanthine determination [109]. The other type of sensor consisted of a microchemical cell that was used as a glucose sensor [110]. The development of the former type of sensor was preceded by that of a micro-oxygen electrode formed on a $2 \times 15 \text{ mm}^2$ silicon chip with a V-shaped groove (made by anisotropic etching of the silicon), a gold cathode and a gold anode (Fig. 3.18.A). The electrodes were formed over a silica layer that acted as insulator. The groove was filled with calcium alginate gel containing 0.1 M aqueous KCl as the electrolyte and covered by a gas-permeable membrane. The oxygen concentration was measured as the reduction current on application of a constant voltage between the two electrodes. The electrode was employed to immobilize autotrophic bacteria in order to construct a sensor for CO_2 (Fig. 3.18.B), which was measured by using the same principle as the microbial sensor with a conventional galvanic oxygen electrode [111]. On addition of a sample solution containing the CO_2 to the buffer where the sensitive area of the sensor was immersed, the carbon dioxide permeated the

The lysine sensor reported by Karube *et al.* was a micro-CO₂ sensor containing immobilized L-lysine decarboxylase (LDC, Fig. 3.18.C). L-lysine catalysed the following reaction



The micro-hypoxanthine sensor was made by immobilizing xanthine oxidase with bovine serum albumin and glutaraldehyde on the micro-oxygen electrode (Fig. 3.18.D). The detection limit achieved was 6.7 μM and the sensitivity was *ca.* 10 times higher than that afforded by conventional hypoxanthine sensors.

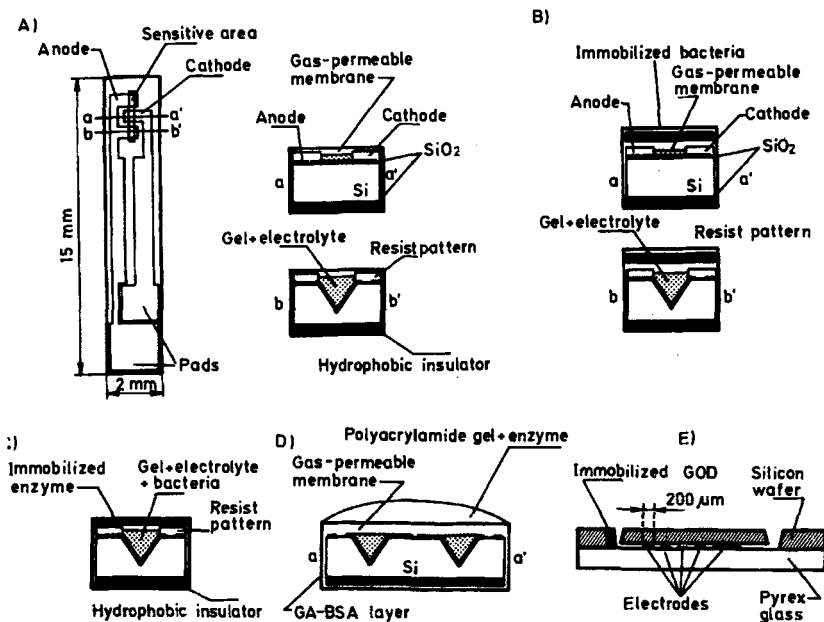


Figure 3.18 — Microbiosensor types. (A) Oxygen electrode. (B) CO₂, (C) L-lysine, (D) hypoxanthine, and (E) glucose biosensors. (Reproduced from [109] and [111] with permission of Marcel Dekker, Inc., and Elsevier Science Publishers).

The disposable micro-glucose sensor consisted of thin-film electrodes positioned on a glass substrate and a small sample chamber (the inner volume of which was only 20 nL) was brought into contact with a silicon chip. Measurements were possible with as little as 1 μL of sample. The sensor structure is depicted in Fig. 3.18.E. The $10 \times 20 \text{ mm}^2$ silicon chip had a V-shaped groove that was 100- μm in wide, 70- μm deep and 5-mm long, in addition to two square sample inlets and five contact holes to connect lead wires to electrodes, all of which were formed by anisotropically etching the silicon. Four working electrodes that were 200 μm in width, and one counter-electrode that was 1.5-mm wide, were formed on a Pyrex substrate. The silicon chip and the Pyrex substrate were thermally bonded.

Table 3.1. Relative response of relevant compounds compared to that of dopamine^(a)

Compound	$i_{\text{P,X}}/i_{\text{P,DA}}$ ^(b)
ascorbic acid	0
acetaminophen	0
adenine	0
catechol	2.01
chlorpromazine	0
L-cysteine	0
dihyronicotinamide adenine dinucleotide (NADH)	0
3,4-dihydroxyphenylacetic acid (DOPAC)	0
3,4-dihydroxyphenylglycol (DOPEG)	0.14
L-dopa	0.06
epinephrine	0.13
homovanillic acid	0
hydroquinone	0.97
norepinephrine	0.23
oxalic acid	0
penicillamine	0
phenol	0
purine	0
riboflavin	0.04
serotonin	0
tyrosine	0
uric acid	0

^(a) Conditions: flow injection measurements at the $1 \times 10^{-4} \text{ M}$ level; differential pulse waveform; scan rate 10 mV/s; pulse amplitude 50 mV; 0.05 M phosphate buffer (pH 4); applied potential -0.2 V ; flow-rate 1.0 mL/min.

^(b) Ratio between the current of each compound over that of dopamine

Glucose oxidase (GOD) was immobilized on the sample inlet by using bovine serum albumin (BSA) and glutaraldehyde. The flow manifold included a buffer carrier line circulated at 0.3 mL/min into which sample volumes of 0.2 μ L were injected [110].

Wang's awareness of the increased stability of biocatalysts in their natural environment materialized in the construction of various enzyme electrodes based on *vegetable biological materials*. His early designs (1988) used non-isolated enzymes and were the basis for his later flow-through sensors, all of which employ enzymes in their natural vegetable environment. Initially, Wang's group used carbon paste which they loaded with green alga *chlorollepyrenoidosa* for the determination of gold [112] and copper [113], or ground banana tissue for quantifying dopamine [114]. This last electrode was incorporated into a flow manifold and used for the determination of the analyte in the presence of potential interferents. The high selectivity of the redox process in the presence of the biological tissue is clearly reflected in Table 3.1, which lists the 22 species assayed and the corresponding interferent-to-analyte peak current ratios [199]. The biosensor performance was substantially improved by using a closely spaced tissue generator electrode-collector pair. The electrode was furnished with a tissue disc and a carbon paste ring that were assembled on a Plexiglas tip machined to be connected to the rotated shaft of a commercially available rotating disc electrode. The plant tissue was press-fitted into a PTFE tube (6.0 mm ID, 6.3 mm OD) that acted as the insulator. The tube containing the vegetable tissue was placed in the centre of a hole (7 mm diameter) drilled through the Plexiglas tip. The gap between the tube and the inner wall was filled with carbon paste, thus forming the collecting ring. Unlike the mixed tissue carbon-carbon electrode [114], dual-electrode assemblies allow independent optimization of the catalytic and sensing functions. The increased selectivity arising from the catalytic effect of the enzymes contained in the plant material used to construct the biosensor was furthered by enzymatic degradation of interferents to non-electroactive species under the working conditions. This concept was illustrated by Wang's group using a zucchini-containing carbon paste electrode. The presence of the enzyme ascorbic acid oxidase (AAO) in the zucchini tissue effectively eliminated the contribution from ascorbic acid, thereby allowing selective measurement of dopamine or norepinephrine. Compared to similar enzyme-based AAO electrodes, the tissue "eliminator" electrode offers high biocatalytic stability and activity at an extremely low cost. The electrode has a useful lifetime of 4 weeks and

allows simultaneous elimination of uric acid by using co-immobilized uricase. Oxygen background currents are eliminated in the presence of ascorbic acid [116]. The rich biocatalytic activity of tissue-containing surfaces has also been exploited for *in situ* enzymatic digestion of interfering protein using a papaya-containing carbon electrode. The presence of the protease enzyme papain in the papaya tissue was found to effectively eliminate protein interferences, as shown in Fig. 3.19, which depicts the response of the plain carbon paste electrode and that of the tissue-modified electrode constructed for the determination of acetaminophen in the presence of various proteins [117].

Several reticulated vitreous carbon (RVC)-plant tissue composite electrodes have also been reported where the open-cell structure of RVC serves as a template for the biocomponent used. One such sensor was constructed by press-fitting 100 pore/in RVC (2-mm thick \times 3 or 6 mm OD) cylinders into a 6-mm diameter cavity of a thin-layer cell. The inner side of the disc was pressed into an edge of a copper wire, which provided electrical contact.

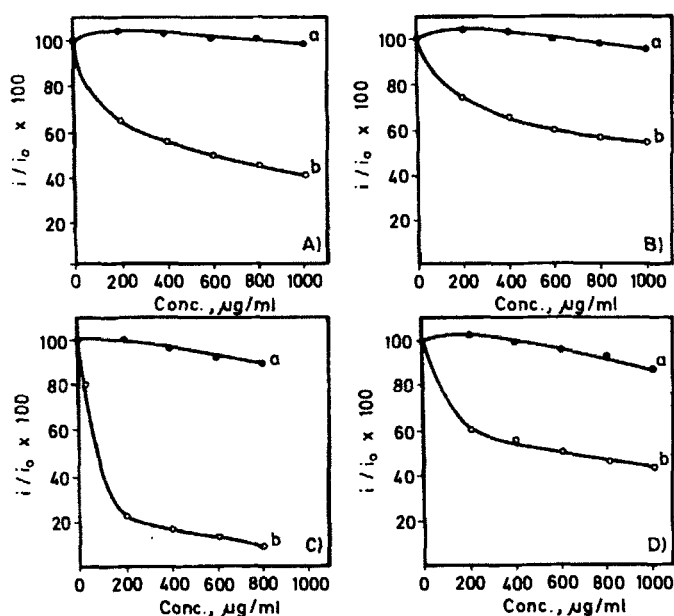


Figure 3.19 — Effect of albumin (A), globulin (B), casein (C), and gelatin (D) on the voltammetric peak of 2×10^{-4} M acetaminophen, at the tissue (*a*) and plain (*b*) carbon-paste electrodes. (Reproduced from [117] with permission of the American Chemical Society).

The biocomponent was then crushed with a mortar and pestle, and packed into the RVC pores, excess biocomponent on the outer face being removed and the surface smoothed with the aid of a spatula. The immediate proximity of the tissue to the carbon surface resulted in a very rapid response, and a high sensitivity was obtained as a result of the high tissue "loading" and large electrode area. These advantages were illustrated with mushroom-, potato- and horseradish root-packed RVC matrices. Tissue-RVC composite electrodes exhibit sigmoidal cyclic voltammograms that are typical of partially blocked surfaces [118].

Ever since the earliest neuronal biosensors based on intact chemoreceptor structures were reported in 1986 [119], analytical dose-response relationships have been demonstrated for a number of natural and synthetic biomolecules [120]. Such "receptrode" biosensors have been shown [121] to exhibit extremely high sensitivity and response times in the millisecond range. They also feature a high selectivity, either inherent in native neuronal receptors [122] or based on the use of a real-time chemometric technique to extract selective responses from mixed neuronal data [123]. Research on neuronal biosensors has been greatly facilitated by the inception of colour video microscope technology for examination and manipulation of neuronal structures under high magnification, as well as production of permanent visual and data records. The above-described combination of video imaging and neurophysiological techniques [124] substantially increases the reliability and convenience of neuronal biosensor construction while extending the scope for experimentation to new sources of chemoreceptor structures. Rechnitz *et al.* reported the first use of neuronal structures from crayfish in biosensor construction and showed them to render satisfactory analytical performance on ordinary tap water (or distilled water) as the analyte medium. Figure 3.20 depicts the experimental set-up used for this purpose. The Plexiglas cell was designed to hold the antennules in place on the mechanical stage of a trinocular dissecting microscope. The cell consisted chiefly of a circular chamber attached to a carrier stream line. The neurobathing chamber (a circular bath) and the carrier stream line were connected to each other by a small hole. Crayfish antennules were mounted in such a way that the nerve fibres were exposed to the neurobathing solution and the chemosensitive portions were brought into contact with the fresh water carrier stream in order to test for potential stimulants. Referenced and ground wires were inserted into the neurobathing chamber. A glass micropipette containing an Ag/AgCl element was also accommodated in the chamber by

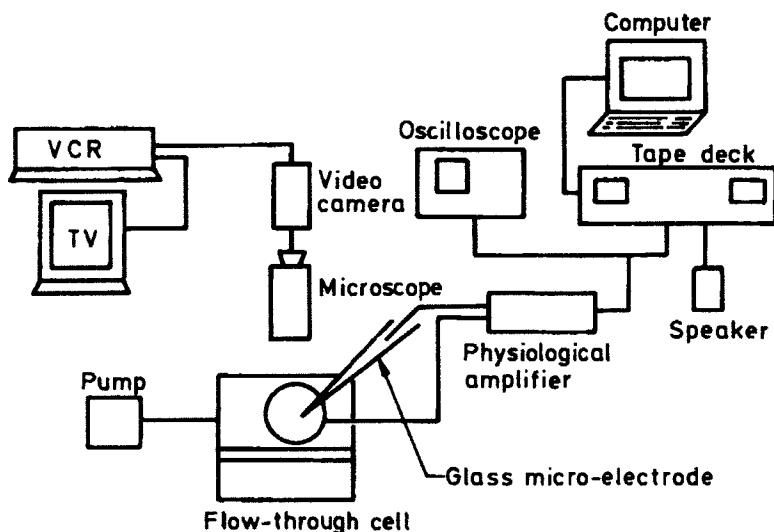


Figure 3.20 — Experimental set-up for implementation of chemoreceptor-based biosensors. (Reproduced from [125] with permission of Elsevier Science Publishers).

means of a micromanipulator in order to measure the action potentials of different nerve cells. A small volume of neurobathing solution was drawn into the micropipette with the aid of a suction syringe in order to create a conducting medium between the Ag/AgCl element and the nerve cells. Once a good seal had been created between the tip of the micropipette and the nerve cells, action potentials were recorded. The peristaltic pump used established a carrier flow and a four-port, two-way valve furnished with a sample injection loop was employed to apply a constant concentration of the stimulus solution to the chemoreceptors for 10 s. The typical lifetimes for these sensors are 8–10 h, depending on the injected analyte concentration. Using fresh water species to construct neuronal biosensors gives rise to increased serviceable lifetimes and diminished interferences [125]. At a later stage, Rechnitz *et al.* developed a reusable neuronal sensor selective to neurotoxin 3-acetaldehyde pyridine. The antennular structure from fresh water crayfish was also used as a sensing element to measure the frequency of action potential responses evoked on chemical stimulation of nerve cells. The sensor reusability was investigated with emphasis on the reproducibility of action potential responses. More recently, these authors reported a neuronal flow-through biosensor using crayfish walking legs. The sensor

responds selectively to pyrazinamide, an antitubercular drug and potent food marker. The sensor was studied in terms of selectivity, the dose–response relationship, reproducibility and serviceable lifetime. Detection of local anaesthetics was accomplished by monitoring the decrease in pyrazinamide-induced firing in the crayfish walking leg. Such a decrease arises from the local anaesthetic block of the axonal sodium channels in the nerves. The sensor's utility for analytical measurements was demonstrated by constructing dose–response curves for several local anaesthetics [127].

Microbial-based biosensors. Microbial biosensors consist of a combination of microorganisms immobilized in a membrane with an electrochemical device, and are suitable for on-line monitoring of biochemical processes. These sensors are either of the respiration activity measurement or the electrochemically active metabolite measurement type. Changes (usually increases) in the respiration activity of microorganisms on assimilation are usually detected by means of an oxygen electrode. The other type of microbial biosensor is normally employed for detecting electrochemically active metabolites such as hydrogen, carbon dioxide, ammonia and organic acids, which are excreted by microorganisms. Most of the microbial sensors reported so far are of the respiration activity measurement type, however.

Microbial sensors offer a number of assets, namely: (a) they are less sensitive to inhibition by solutes and more tolerant to suboptimal pH and temperature values than are enzyme electrodes; (b) they have longer lifetimes than enzymes; and (c) they are less expensive than enzyme electrodes as they require no active enzyme to be isolated. On the other hand, they lag behind enzyme electrodes in a few other respects; thus, (a) some have longer response times than their enzyme counterparts; (b) baseline restoration after measurement typically takes longer; and (c) cells contain many enzymes and due care must be exercised to ensure adequate selectivity (*e.g.* by optimizing the storage conditions or using specific enzyme reactions) —some mutant microorganisms lack certain enzymes.

Microbial species employed to construct sensors are usually immobilized by membrane or gel entrapment.

Microbial sensors usually possess long lifetimes, yet proper maintenance is mandatory. Thus, the overall activity of immobilized microorganisms should be kept as constant as possible. For this purpose, sensors should be stored in phosphate buffer containing no nutrients at 4°C in order to avoid microorganism growth in the membrane. If the sensor activity diminishes for some reason, then the sensor should be dipped into a nutrient medium

for as long as required for activity to be regained by growth of new cells. Also, care should be exercised to avoid contamination and hence impaired sensitivity and selectivity; this is even more critical than with enzyme

Table 3.2. Microbial sensors

Analyte	Immobilized microorganism	Device type	Response time (min)	Range (mg/L)
Assimilable sugars	<i>Brevibacterium lactofermentum</i>	O ₂ probe	10	10–200
Glucose	<i>Pseudomonas fluorescens</i>	O ₂ probe	10	2–20
Acetic acid	<i>Trichosporon brassicae</i>	O ₂ probe	10	3–60
Ethanol	<i>Trichosporon brassicae</i>	O ₂ probe	10	2–25
Methanol	Unidentified bacteria	O ₂ probe	10	5–20
Formic acid	<i>Citrobacter freundii</i>	Fuel cell	30	10–10 ³
Methane	<i>Methylomonas flagellata</i>	O ₂ probe	2	0–6.6 ^(a)
Glutamic acid	<i>Escherichia coli</i>	CO ₂ probe	5	8–800
Cephalosporin	<i>Citrobacter freundii</i>	pH electrode	10	10 ² –5×10 ²
BOD	<i>Trichosporon cutaneum</i>	O ₂ probe	15	3–60
Lysine	<i>Escherichia coli</i>	CO ₂ probe	5	10–10 ²
Ammonia	Nitrifying bacteria	O ₂ probe	10	0.05–1
Nitrogen dioxide	Nitrifying bacteria	O ₂ probe	3	0.51–255 ^(b)
Nystatin	<i>Saccharomyces cerevisiae</i>	O ₂ probe	1 h	0.5–54 ^(c)
Nicotinic acid	<i>Lactobacillus arabinosis</i>	pH electrode	1 h	10 ⁻⁵ –5
Vitamin B ₁	<i>Lactobacillus fermenti</i>	Fuel cell	6 h	10 ⁻³ –10 ⁻²
Cell population	—	Fuel cell	15	10 ⁸ –10 ^{9(d)}
Mutagen	<i>Bacillus subtilis</i>	O ₂ probe	1 h	1.6–2.8×10 ³

^(a) mmol ^(b) µg/mL ^(c) unit/mL ^(d) number/mL

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biosensors since use of antibacterial agents such as sodium azide or an antibiotic is excluded.

Table 3.2 lists the most salient microbial sensors reported to date, together with the type of immobilized microorganism and measurement used, and the response time and dynamic range achieved in each instance. As can be seen, most of these biosensors rely on amperometric measurements. Some of them are described in detail below.

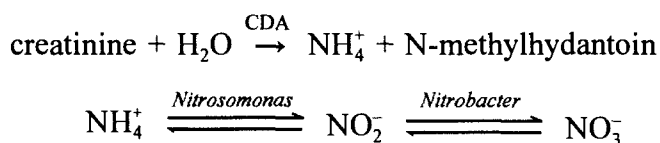
Biochemical oxygen demand (BOD) is one of the most widely determined parameters in managing organic pollution. The conventional BOD test includes a 5-day incubation period, so a more expeditious and reproducible method for assessment of this parameter is required. *Trichosporon cutaneum*, a microorganism formerly used in waste water treatment, has also been employed to construct a BOD biosensor. The dynamic system where the sensor was implemented consisted of a 0.1 M phosphate buffer at pH 7 saturated with dissolved oxygen which was transferred to a flow-cell at a rate of 1 mL/min. When the current reached a steady-state value, a sample was injected into the flow-cell at 0.2 mL/min. The steady-state current was found to be dependent on the BOD of the sample solution. After the sample was flushed from the flow-cell, the current of the microbial sensor gradually returned to its initial level. The response time of microbial sensors depends on the nature of the sample solution concerned. A linear relationship was found between the current difference (*i.e.* that between the initial and final steady-state currents) and the 5-day BOD assay of the standard solution up to 60 mg/L. The minimum measurable BOD was 3 mg/L. The current was reproducible within 6% of the relative error when a BOD of 40 mg/L was used over 10 experiments [128].

The agar diffusion method is the typical choice for bioassays of antibiotics, but is unsuitable for antibiotics consisting of heterogeneous mixtures of closely related compounds, which are scarcely soluble in water and diffuse poorly in agar gel. In addition, those antibiotics that are unstable in bright sunlight and produce zones of inhibition that may be neither clear nor proportional in size to the logarithm of the antibiotic concentration are difficult to assay by this method.

A novel bioassay for nystatin based on the use of a microbial sensor was recently reported. Nystatin is believed to bind to the sterol present in the membranes of sensitive cells, leading to the formation of pores. The subsequent death of the microorganism is preceded by leakage of cellular materials. Microbial death can be detected by means of an oxygen electrode.

Based on this principle, a yeast electrode consisting of a membrane supporting immobilized yeast (*Saccharomyces cerevisiae*) cells was attached to an oxygen electrode and covered with a collagen membrane in order to prevent loss of yeast cells. Nystatin was dissolved in dimethylformamide (DMF) and diluted to an appropriate concentration with 0.05 M phthalate buffer of pH 4.5 containing 500 mg/L glucose. The DMF concentration in the sample solution must be kept below 0.66%. The nystatin concentration was measured from the rate of current increase since dying cells consumed less oxygen. Concentrations above 0.5 unit nystatin/mL could thus be readily measured [129].

One of the pitfalls of microbial sensors, viz. their low selectivity, can be overcome by combining cells with an immobilized enzyme. Thus, creatinine deaminase (CDA, EC 3.5.4.21) hydrolyses creatinine to N-methylhydantoin and ammonium ion, the ammonia produced being successively oxidized to nitrite and nitrate ion by nitrifying bacteria. These bacteria have not yet been characterized but are known to be a mixed culture of *Nitrosomonas* sp. and *Nitrobacter* sp. The reaction sequence involved is as follows:



The bacteria consume oxygen during ammonia oxidation, so oxygen depletion can be detected by using an oxygen electrode. A combined creatinine sensor thus consists of a cellulose dialysis membrane, immobilized creatinine deaminase, immobilized nitrifying bacteria and an oxygen electrode [130].

3.2.1.2.2 Potentiometric flow-through biosensors

Though not so widely developed as their amperometric counterparts, potentiometric flow-through sensors make a fairly large group of sensors comprising both laboratory-made and commercially available designs. Ever since the first potentiometric biosensor was developed in 1969 [131], over one hundred devices of this type have been reported. Such assets as instrumental simplicity (in contrast to amperometric probes, which require use of a polarographic system, potentiometric biosensors can be constructed from an ordinary pH-meter), in addition to low cost and the ready availability of a variety of reliable ISEs, make potentiometric biosensors particularly attractive. Sensors based on potentiometric measurements can be classified into two groups, namely: (a) those constructed from a selective

electrode, which obviously include the pH glass electrode and redox-based electrodes; and (b) those made from field effect transistors. Sensors in the latter group can in turn be classified according to whether they consist of a conventional electrode that responds selectively to some product of an enzyme reaction or use pH-responsive field effect transistors (FETs).

The classic potentiometric enzyme electrode is a combination of an ion-selective electrode-based sensor and an immobilized (insolubilized) enzyme. Few of the many enzyme electrodes based on potentiometric ion- and gas-selective membrane electrode transducers have been included in commercially available instruments for routine measurements of biomolecules in complex samples such as blood, urine or bioreactor media. The main practical limitation of potentiometric enzyme electrodes for this purpose is their poor selectivity, which does not arise from the biocatalytic reaction, but from the response of the base ion or gas transducer to endogenous ionic and gaseous species in the sample.

Gas- and ion-selective biosensors. A number of bioprobes use enzymes that release ammonia, which can be detected by means of a gas- or ion-selective electrode. In the former case, large background levels of endogenous ammonia nitrogen (total ammonia plus ammonium ion) in the sample may lead to positive errors in measurements of a given substance. An ammonium-selective electrode based on nonactin as the membrane ionophore may be interfered by both endogenous ammonium and potassium when used as a transducer. Meyerhoff *et al.* used an anion-exchange perfluorinated membrane in conjunction with a non-equilibrium flow-injection measurement assembly to reduce endogenous ionic interferences, which facilitated use as a flow-through detector for direct measurements of analyte substrates in complex samples [132]. Other biosensors based on ammonia detection are applied to substrates that can release gaseous NH_3 in a reaction catalysed by a suitable enzyme immobilized directly in the sensing membrane. Such substrates as urea [133], creatinine [134] and several aminoacids [135–137] have been determined in this way. The first potentiometric enzyme sensor for direct assay of dissolved methionine was reported by Guilbault *et al.* [137] and used immobilized methionine-lyase in the NH_3 electrode membrane. The immobilized enzyme remained stable for at least 3 months and exhibited a linear response to methionine over the concentration range 10^{-5} to 10^{-2} M with no interference from ordinary aminoacids.

Karube *et al.* have constructed several gas-sensing devices by using immobilized microorganisms and a potentiometric transducer. Two of them

were used as microbial sensors for methane and carbon dioxide. The latter uses autotrophic bacteria and is currently available in commercial form. However, the potential of the inner pH electrode and the gas-permeable membrane of the CO₂ electrode are effected by various ions and some organic and inorganic volatile acids. Also, the precision of the conventional potentiometric CO₂ electrode is somewhat limited by the Nernstian type of response it provides [138].

pH measurement-based biosensors. The pH glass electrode is widely available and used. The fact that many enzyme reactions give rise to pH changes is no doubt one reason for the vast amount of effort devoted to constructing enzyme-based pH sensors, the most serious pitfall of which is probably that arising from the sample buffering capacity [139]. This is particularly true with real samples (*e.g.* biological fluids, fermentation broths), which usually have a high buffering capacity, so oscillations in the pH with time or from sample to sample are prone to occur. Variations in the pH can be addressed by making differential measurements with the aid of a second, analogous electrode containing no immobilized enzyme. One such differential pH-meter enabling changes in the medium pH as small as 1×10^{-4} units to be recorded was successfully applied to biological samples [140]. Detecting slight pH changes within a biological layer usually calls for a low buffering power. This can be achieved by diluting the sample, which introduces some risk of contamination and loss of sensitivity. In addition to pH glass sensors, various metal–metal oxide electrodes have been advantageously used in combination with enzymes. The pH change within a biological layer arising from an enzyme reaction is believed to affect the structure of the oxide layer and hence alter the electrode potential [141]. Antimony oxide [141–143], palladium oxide [144] and titanium electrodes coated with an oxide (IrO₂ or RuO₂) [145] have been shown to excel the classic pH glass electrode in terms of response time, robustness and miniaturizability.

Mottola *et al.* reported a special biosensor based on nylon shavings onto which an enzyme was covalently bonded, a flat-surface combination pH electrode for proton monitoring and a flow-cell accommodating the enzyme–support suite in the bottom. On placing the electrode with its surface parallel to the cell bottom, the support–enzyme suite was pressed onto it. The device exhibited good diffusional and partition features that resulted in a relatively fast response, good stability, operational simplicity, low sample and reagent consumption and a high sampling frequency. It was successfully

used to immobilize urea and determine it in physiological salt solutions [146].

Redox-based biosensors. Noble metals (platinum and gold) and carbon electrodes may be functionalized by oxidation procedures leaving oxidized surfaces. In fact, the potentiometric response of solid electrodes is strongly determined by the surface state [147]. Various enzymes have been attached (whether physically or chemically) to these pretreated electrodes and the biocatalytic reaction that takes place at the sensor tip may create potential shifts proportional to the amount of reactant present. Some products of the enzyme reaction that may alter the redox state of the surface (*e.g.* hydrogen peroxide and protons) are suspected to play a major role in the observed potential shifts [147].

The operational principle of a *biochemical field-effect transistor* (bioFET) is the determination of a pH shift produced by an enzyme reaction in an enzyme-loaded membrane covering the pH-sensitive gate area of the FET. The extent of the shift depends on the substrate concentration present. The vast amount of research that has gone into these enzyme-modified field-effect transistors [139] has unfortunately not met with a proportional number of applications [148]. Reinhardt *et al.* developed several such sensors for the determination of glucose, urea, penicillin G and V, and cephalosporin C, which they implemented in suitable flow injection manifolds. All the sensors were constructed by covering the pH-sensitive gate areas of ion-selective field-effect transistors with enzyme membranes by using the following procedure: a small drop of enzyme solution was placed on the FET gate and the FET was spun at 5000 rpm in order to form a thin, even film, which was allowed to dry for 15 min and then soaked in 370 μL of an immobilization solution containing 0.1 mL of glutardialdehyde, 1 mL of water and 1.75 mL of isopropyl alcohol. After a further 15 min, the FET was washed with distilled water and allowed to stand in a phosphate buffer of pH 8 for 1 h prior to use. These biosensors exhibit excellent properties including measuring ranges of 1–2 orders of magnitude of substrate concentration, a stable response for 3–12 weeks (depending on the type of enzyme used), and a short delay time that allows up to 15–20 measurements per hour to be made. These features allow use in bioprocess monitoring with results that compare favourably with those obtained in off-line liquid chromatographic determinations [149,150].

A biosensor of this type was reported by Shiono *et al.* and used for the determination of urea [151]. The probe consisted of two hydrogen ion-

sensitive elements from an epitaxially grown silicon wafer (Fig. 3.21.A). Figure 3.21.B depicts the structure of the enzyme-modified sensor. The response obtained by incorporation into the flow-cell shown in Fig. 3.21.C resulted in a linear determination range for the analyte from 0.25 to 50 $\mu\text{g/mL}$ urea, with an RSD less than 3%.

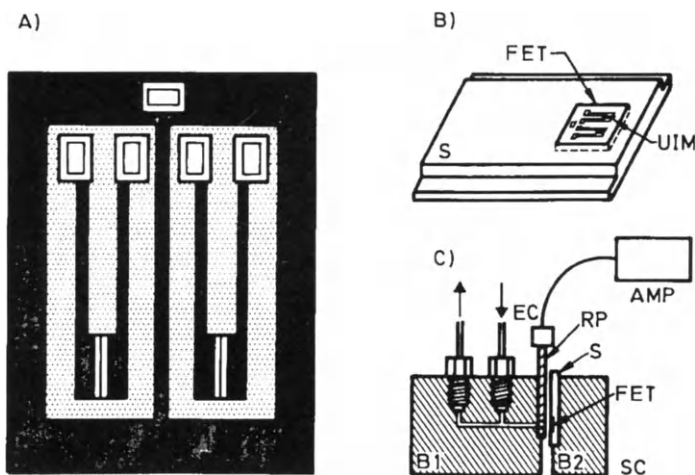


Figure 3.21 — (A) Integrated FET with two hydrogen ion-sensitive FET elements. (B) Structure of enzyme-modified FET sensor: S plastic card; FET enzyme-modified FET chip; IUM, immobilized urease membrane. (C) Flow-through cell: B1 fixed sensor cell block; B2 movable sensor cell block; SC flow-through cell; EC electrical connector; RP silicone rubber sheet; AMP amplifier. (Reproduced from [151] with permission of Elsevier Science Publishers).

3.2.1.3 Flow-through calorimetric biosensors

Any (bio)chemical reaction is accompanied by energy conversion, most often in the form of heat production, the amount of heat produced being proportional to that of substance converted. Therefore, heat is a highly non-specific expression of a (bio)chemical reaction but can be used as indicative for a given substance when this is selectively converted (*e.g.* by effect of a catalyst, particularly an enzyme). This section discusses three types of sensors based on the use of as many types of devices for measurement of the heat involved in a biochemical reaction, namely: fibre optics, polymer films and thermistors.

Fibre optic-based flow-through calorimetric sensors. Most of many current uses of fibre optics for detection involve extrinsic sensors. However, some are used as intrinsic acoustic, magnetic, thermal and pressure sensors, among others [152–155]. One of such less common sensors is a fibre optic-based enthalpimeter. Changes in the light propagation properties of a fibre caused by temperature arise from thermally induced length changes, the temperature dependence of the core refractive index, and elasto-optic changes in the core due to thermally induced stress–strain changes. The instrument used to measure such changes (Fig. 3.22.A) comprises a two-arm Mach–Zehnder interferometer constructed from mono-mode fibre optic waveguides. One of the interferometer arms is coated with an immobilized enzyme while the other is used as a reference. Both arms are firmly held in the middle of a conduit that is incorporated into an FI system. A common source of phase coherent light is launched down both arms. The exit beams from the two fibres are superimposed in the far field of a linear array optical detector, which produces the classical two-slit pattern of light and dark bars due to interference. As sample plugs containing specific substrates for the enzyme pass the sensing area, heat is produced around one fibre. Such heat partitions between the flowing stream and the fibre and produces a thermal and stress–strain effect on the coated fibre that changes its light propagation characteristics. Even though shifts in the resulting bar patterns can indeed be detected by using a single optical detector, a linear array has a number of advantages. If an integral number of periods of the bar pattern, n , illuminate the active area of the array, a Fourier transform of the data from the canned array will produce a new real and imaginary data set. The ratio of the real and imaginary values at the spatial frequency n gives the phase-angle difference between the two beams and simultaneously eliminates noise contamination, which is found at the other spatial frequencies. The phase-angle difference in turn can be related to the sample concentration.

Studies of the peroxidase/catalase system involving this type of biosensor provided response curves with rather ill-defined maxima and badly drifting baselines. The origin was probably in the bubbles of gaseous oxygen produced at late reaction stages. The bubbles clung to the fibre, thereby seemingly introducing mechanical and/or thermal effects that distorted the expected profile. Although addition of a surfactant (ethoxylated lauryl alcohol) lessened distortion and smoothed the baseline, the ill-defined peaks did not respond to peak height or area measurements. The peaks were broad, did not return to any reliable baseline position, and were characterized by

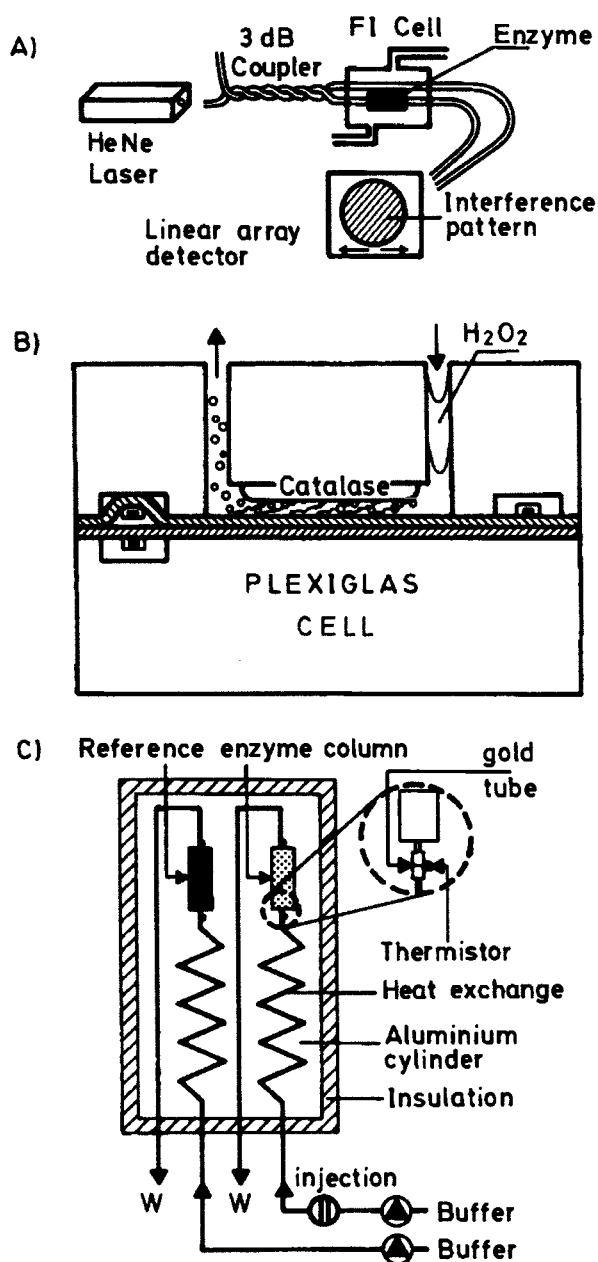


Figure 3.22 — (A) Scheme of the Mach-Zehnder interferometer/enthalpimetric. (Reproduced from [152] with permission of Elsevier Science Publishers). (B) Cross-section of polyvinylidene fluoride film-based enthalpimetric. (Reproduced from [156] with permission of the American Chemical Society). (C) Schematic diagram of an enzyme thermistor.

low signal-to-noise ratios. Deriving consistent area or height or standard peak-picking algorithms proved impossible. Reliable quantitative calibration curves could only be derived by evaluation of the slope of the phase-angle difference curve as a function of time at a fixed arbitrary point near the beginning of heat evolution. These fibre-optic sensor systems are extremely sensitive to environmental noise, so they typically require pneumatically supported optical tables. Pressure fronts developed from droplets falling from the exit of the FI tubing are often seen. Care is required in mounting the fibres in the FI channel. Diminished response or even reversal of the signal response is easily induced by improper tensioning. Heat-induced stress competes with the radial and axial stresses resulting from the fixing of the two ends of the sensor area within the flow-cell. Heat may increase or decrease the total stress in the system. One crude analogy is the effect of heat on a piece of metal fixed at both ends. Notwithstanding their limitations, these sensor systems may have useful potential applications provided they are properly engineered to circumvent them [156].

Polymer film-based flow-through calorimetric sensors. Modern flow systems place unusual constraints on sensor configuration. Developing rugged, inexpensive sensors that can readily be adapted to the geometries imposed by FI systems is essential. In this respect, fabricating a useful enthalpimetric biosensor from films of piezo- and pyroelectric organic polymers might be an effective approach [157]. These materials are thin enough for simple cutting and moulding to allow the sensor material to be conformed to the required geometry of the sample cell rather than designing the cell geometry around a fixed sensor. The films needed for this purpose are inexpensive and available from several sources. For example, polyvinylidene fluoride (PVDF) films ranging in thickness from a few microns to 1 mm can be made piezo- and pyroelectric by special treatment after manufacturing [158]. The materials are simultaneously subjected to a high temperature, elongation stress along one or two axes and a high direct current field potential. As the material is allowed to cool, with the stress and polling field applied, orientation effects arise that result in residual polarization of the field rendering it piezo-pyroelectric in such a way that it exhibits electrostriction. In principle, a biosensor can be fabricated from the material by immobilizing an enzyme on one surface and flowing a substrate solution past the film sensor. A simple charge amplifier can be used to measure the difference between the two sensor surfaces. PVDF films of 40 μm were used to design a sensor based on this methodological principle.

These films have a thin metal coating that is vapour-deposited on each surface to facilitate the electrical polling process and can be used to make electrical contact in sensor applications.

Film of this type was configured as a bimorph or double laminate by placing like surfaces of two pieces face to face. The bimorph was pressure-mounted in an FI cell made of Plexiglas (Fig. 3.22.B) with three electrical contact points: the mating inner surfaces of the bimorph and the two outer surfaces. The enzyme was immobilized onto the channel roof. Attempts at placing the enzyme directly onto the sample film surface led to poorer response as regards both signal and noise level. This was a result of poor thermal transport characteristics in the hydrated biopolymer. The signals from the sample and reference surfaces were first buffered via the non-inverting inputs of the two electrometers, which had very high input impedance and low input bias currents. The outputs of the two electrometers were used as inputs to an instrument amplifier with a 25- μ s successive approximation, and noise was reduced by using a digital filter. Quantitation was approached in various ways, the best results being provided by straightforward peak-height measurements.

This type of sensor was also assessed by using the peroxidase/catalase system and an injected sample volume of 80 μ L. The calibration curve run was linear from 0.004 to 1.0 M. Modelling of the heat transport within the film showed that the thermal transport process can be treated as a semi-infinite solid (*i.e.* an infinitely thick solid with one surface). The model produced thermal time gradients that were consistent with those experimentally obtained, and satisfactorily predicted the timing and amplitude of the phase change observed as the thermal boundary passed from one layer of the bimorph to the other. The modelling suggested that some improvement might be achieved by inserting a thermal insulator between the two layers of the bimorph [156].

Thermistor based flow-through calorimetric sensors. Enzyme thermistors make the most widely developed type of heat measurement-based sensors. The thermistors are normally used as temperature transducers in these devices. Thermistors are resistors with a very high negative temperature coefficient of resistance. They are ceramic semiconductors made by sintering mixtures of metal (manganese, nickel, cobalt, copper, iron) oxides. Like the two previous groups, thermistor sensors do not comply strictly with the definition of "sensor" as they do not consist of transducers surrounded by an immobilized enzyme; rather, they use a thermistor at the end of a small

enzyme reactor inserted in a flow system —often, the denomination is applied to the entire experimental set-up, including a thermostat and a reference for compensation of non-specific heat production (e.g. from friction, adsorption, desorption and turbulence effects). Figure 3.22.C illustrates the operation of a typical enzyme thermistor. In a thermostat (stable to within 0.01°C), a buffer circulated at a flow-rate of $0.5\text{--}5\text{ mL/min}$ is temperature-equilibrated with its surroundings and then passed through the "enzyme reactor", at the end of which the thermistor is glued with heat-conducting epoxy resin to a gold capillary. The reference (a selected thermistor of the same characteristics as the previous one) is either mounted on the end of a similar column containing no enzyme, the entrance to the enzyme column, or the inner wall of the thermostat. The signal is produced from adjustment of the resistances through a Wheatstone bridge, and can be as high as 100 mV (recorder input) for a temperature change of 0.01°C . Since the enthalpy of enzyme-catalysed reactions is typically $30\text{--}100\text{ kJ/mol}$, and amount of substance of 10^{-7} mol , equivalent to 0.1 mL of a 10^{-3} M solution, will give rise to a maximum temperature rise of 0.2°C provided there is no dilution, no heat loss and full conversion of the substance concerned by the enzyme. In practice, *ca.* 50% of the calculated maximum value is more realistic. Ideally, a thermistor device would allow differences of 0.001°C to be measured; however, because of the above-described disturbances, temperature differences of less than 0.005°C must usually be accepted as measuring signals. This means that the lower detection limit for a substrate under the above-mentioned experimental conditions would be 10^{-5} M .

Even though most enzyme thermistors have been used for determining substrates, a few applications to the determination of inhibitors and activators, as well as enzyme activities, have also been reported. The enzymes employed for this purpose are usually isolated previously, though some are used in their original tissues and microorganisms [157].

3.2.1.3.1 Enzyme immobilization

Many of the fairly large number of enzyme thermistor biosensors reported so far have been used for the determination of biological substrates or, to a much lesser extent, inorganic substrates. Experimental set-ups similar to that depicted in Fig. 3.22.C were used to determine the substrates listed in Table 3.3, which also gives the primary enzymes and any auxiliary enzymes or reagents employed to improve the determination [158].

Table 3.3. Determinations of substrates by use of enzyme thermistor biosensors

Analyte	Main enzyme	Auxiliary enzyme or reagent (effect)
Alcohols	Alcohol oxidase	Catalase (increases stability of main enzyme on immobilization)
Ascorbic acid	Ascorbate oxidase	Concanavalin A-conjugated Sepharose (facilitates enzyme replacement or renewal)
ATP/ADP	Apyrase (ATPase + ADPase)	
Cellobiose	Glucose oxidase + catalase	β -Glucosidase
Cephalosporins	Cephalosporinase	β -Lactamase (enhances activity of main enzyme)
Cholesterol	Cholesterol oxidase	
Creatinine	Creatinine deiminase	
Galactose	D-Galactose oxidase	
Glucose	Glucose oxidase	Catalase (increases stability of immobilized glucose oxidase) Benzoquinone (electron acceptor)
Hydrogen peroxide	Catalase or peroxidase	
L-Lactate	Lactate-2-monooxygenase or lactate oxidase	
L-Malate	Malate dehydrogenase	
Oxalate	Oxalate decarboxylase or oxalate oxidase	
Penicillin G and V	β -Lactamases	
Phospholipids	Phospholipase/choline oxidase	Catalase (increases stability of main enzyme)
Sucrose	Invertase	
Triglycerides	Lipoprotein lipase	
Urea	Urease	
Uric acid	Uricase	
Xanthine/hypoxanthine	Xanthine oxidase	
Pyrophosphate	Inorganic pyrophosphatase	Tris buffer

(Reproduced from [6] with permission of Marcel Dekker, Inc.)

The heat produced by enzyme reactions yielding or consuming protons (*e.g.* those involving proteolytic enzymes) can be substantially increased by using a buffer with a high protonation enthalpy such as Tris (-47.5 kJ/mol compared to only -4.7 kJ/mol for an ordinary phosphate buffer).

Satoh and Ishii developed an enzyme thermistor sensor for the determination of inorganic pyrophosphate [159] by immobilizing inorganic pyrophosphatase on controlled pore glass and using it to load a plastic column of 0.28 mL packing volume and 7 mm ID that was mounted in a Plexiglas holder comprising two channels (one of which contained a small glass-encapsulated thermistor fixed on a gold capillary at the column outlet while the other channel contained the carrier stream). The assembly was placed in a thermostated aluminium cylinder accommodating two heat exchangers. Two identical temperature probes were inserted; one column contained the immobilized enzyme preparation and the other washed glass beads only. Enthalpy changes resulting from pyrophosphate hydrolysis are very small (-5 kcal/mol) and almost all of the evolved heat is related to the transfer of hydrogen ions to an appropriate buffer. Chemical amplification was achieved by using Tris buffer, which, as stated above, has a heat of protonation 10 times higher than that of phosphate. Injection of 0.5-ml samples gave responses corresponding to *ca.* 40% of the steady-state signals, which returned to the baseline within 4 min from the initial rise of the peak, and the baseline was 1% of full scale. The sensitivity was increased by injecting sample volumes up to 2.5 mL, but increasing the volume to 5.0 mL made no further difference to the signals provided by 0.5–5.0 mM pyrophosphate. The calibration graph was linear from 0.1 to 20 mM pyrophosphate and the RSD was 2.0% ($n = 30$). Up to 500 assays could be performed with no appreciable loss of enzyme activity.

The overall temperature signal can also be increased by using sequentially acting enzymes. This commonly involves hydrolytic enzymes such as disaccharide splitting enzymes, which can be combined with an enzyme acting on the resulting monosaccharide. One typical combination is that between β -galactosidase and glucose oxidase/catalase for the determination of lactose.

Amplification of the sensitivity of substrate or co-enzyme recycling is especially efficient in thermometric analysis since all the reactions involved frequently contribute to increasing the overall temperature change. One case in point is the determination of lactate or pyruvate by substrate recycling using co-immobilized lactate oxidase and lactate dehydrogenase [160].

L-lactate is oxidized by lactate oxidase to pyruvate, which is reduced back to lactate by LDH. The total enthalpy change for this system can be further increased by addition of catalase, which makes the overall enthalpy change as large as -225 kJ/mol, so signal increases greater than 1000-fold can be obtained as a result. Co-enzyme recycling was also used for the determinations of ATP/ADP [161] and NAD(H) [162].

Methods based on the inhibitory effect of the analyte and the use of an enzyme thermistor have primarily been applied to environmental samples and typically involve measuring the inhibitory effect of a pollutant on an enzyme or on the metabolism of appropriate cells [162]. The inhibiting effect of urease was used to develop methods for the determination of heavy metals such as Hg(II), Cu(II) and Ag(I) by use of the enzyme immobilized on CPG. For this purpose, the response obtained for a 0.5-mL standard pulse of urea in phosphate buffer at a flow-rate of 1 mL/min was recorded, after which 0.5 mL of sample was injected. A new 0.5-mL pulse of urea was injected 30 s after the sample pulse (accurate timing was essential) and the response compared with that of the non-inhibited peak. After a sample was run, the initial response could be restored by washing the column with 0.1–0.3 M NaI plus 50 mM EDTA for 3 min. Under these conditions, 50% inhibition (half the initial response) was obtained for a 0.5-mL pulse of 0.04–0.05 mM Hg(II) or Ag(I), or 0.3 mM Cu(II). In some cases, the enzyme was inhibited irreversibly. In this situation, a reversible enzyme immobilization technique

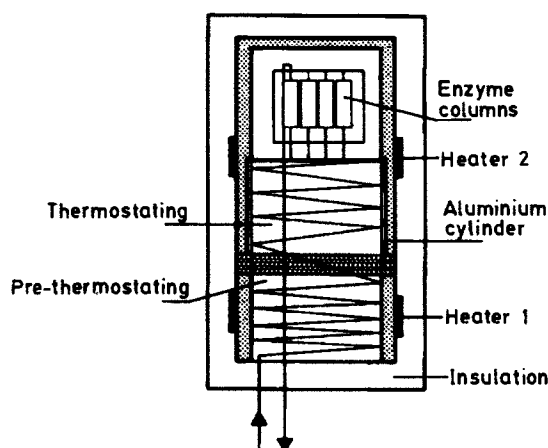


Figure 3.23 — Schematic diagram of a four-channel enzyme thermistor sensor. (Reproduced from [164] with permission of Elsevier Science Publishers).

allowing easy replacement of depleted enzyme was required. This was accomplished by reversibly binding cholinesterase (EC 3.1.1.7) to a concanavalin A–Sephrose column [162]. Used enzyme was removed by injecting a pulse of 0.2 M glycine/HCl at pH 2.2. Fresh enzyme was thus immobilized on the column simply by injecting the enzyme preparation while the column remained in the enzyme thermistor apparatus.

Many enzymes require the presence of metal ions at their active sites to be catalytically active. In many cases, the metal ion can be removed by using a strong chelating agent. The resulting apoenzyme is inactive but can be reactivated by exposure to a sample containing an appropriate metal ion. This measuring principle was used to develop calorimetric determinations for a variety of metal ions. There are affinity biosensors of this type available for Zn(II), Co(II) and Cu(II), among other metals [163].

3.2.1.3.2 *Microorganism immobilization*

The advantages of microorganisms as catalysts have also been exploited for developing enzyme thermistors. The early tests of Mosbach and Danielsson for the determination of glycerol by use of a thermistor containing *Gluconobacter oxydans* immobilized on calcium alginate [162] led to a wider approach reported by Hundek et al., who used a four-channel enzyme thermistor system for biotechnological process monitoring and control [164]. They employed glucose oxidase and catalase for the determination of glucose, invertase covalently bound to oxirane acrylic supports for maltose, and *Saccharomyces cerevisiae* immobilized on calcium alginate for monitoring assimilable sugars. The experimental set-up used is depicted in Fig. 3.23 and allowed up to four analytes to be quantified simultaneously. Signal detection was facilitated by a microprocessor control unit. The user was allowed to define the determination channels via the software and then choose a reference column or measurement between two thermistors of the same column or other special measurement applications. The system was used to monitor and control cultivation of *Cephalosporium acremonium* and *Bacillus licheniformis*.

3.2.1.3.3 *Determination of enzyme activities*

Enzyme thermistors can be altered for measuring the activity of soluble enzymes. For this purpose, an inactive or empty Teflon column can be used as a reaction chamber. The sample and a buffer containing a suitable substrate in excess are passed through heat exchangers and thoroughly

mixed. The mixture is then passed through a short heat exchanger to dissipate mixing and solvation heats. In the enzyme thermistor proposed by Danielsson *et al.* [165], each flow channel includes one long heat exchanger tube in contact with an outer aluminium jacket and one short tube in contact with an inner heat sink. These facilitate the determination of enzyme activities. Residence times shorter than 1 min are long enough for enzyme activities down to 0.01 U/mL to be determined. The procedure is quite general and applicable to both sample pulses and continuously introduced samples. The system is particularly effective for monitoring enzyme purification processes, but can equally be used for direct continuous-flow detection of crude samples by using inexpensive substrates to control downstream processing.

One alternative method for the determination of enzyme activities which is particularly effective at low enzyme concentrations involves enrichment with the enzyme by affinity binding (preferably of the reversible type) to an affinity column in the enzyme thermistor unit. The enzyme activity is determined by introducing a pulse of excess substrate.

3.2.1.4 Flow-through piezoelectric sensors

The principle behind piezoelectric sensors is the linear relationship between the change in the oscillating frequency of a piezoelectric crystal and the mass variation on its metal surface. The piezoelectric effect arises from the fact that some crystals contain positively and negatively charged ions, which separate under stress. This sets the crystal atoms in motion and the motion results in separation of charge sites, *i.e.* in polarization. This cycle of oscillating strain gives rise to an oscillating electrical field, the frequency of the electrical oscillation being identical with the vibration frequency of the crystal. Also, placing a piezoelectric crystal in an oscillating field causes it to vibrate at the same frequency as the oscillating field. This energy transfer from the electrical field is very inefficient except when the frequency of the oscillating electrical field is the same as the resonant frequency of the crystal. This effect can be exploited by incorporating quartz crystals into oscillator circuits, the frequency of the entire circuit becoming the resonant frequency of the quartz crystal. Based on the same principle, the resonant frequency at a given crystal (*e.g.* one used in a biosensor) can be determined from the frequency of an electrical oscillator circuit of which the crystal is a component.

The general approach to exploiting the piezoelectric effect involves coating a piezoelectric crystal with a material that interacts highly selectively with the target molecule. On exposure of the crystal to the substance of interest, adsorption occurs which produces a frequency change that can be used to measure the amount of material adsorbed. The coating material used determines the selectivity of the resulting piezoelectric sensor. Biologically active materials such as antibodies, enzymes and antigens are much more selective than are organic and inorganic coating materials. The selectivity provided by biological catalysts was first exploited by Clark and Lyons in 1962 to build an enzyme-based biosensor for glucose [166]. Even though such a biosensor could in principle be applied to both liquid and gaseous samples, few attempts at dealing with the latter have so far been reported, of which the sensors for formaldehyde [167] and cocaine [168] developed by Guilbault *et al.* deserve special mention. The sensors described below are all liquid-phase piezobiosensors.

Figure 3.24 depicts a piezoelectric sensor consisting of two oscillator circuits: a detector crystal oscillator and a reference crystal oscillator. The two are identical except for the fact that the reference oscillator is not coated with biological material and is intended to correct for temperature and humidity fluctuations, as well as other interfering effects. The two oscillator frequencies are fed to a mixer that provides the difference in frequency between the two crystals. In order to use the piezoelectric effect to detect a target dissolved substrate it should be reacted with a suitable biocatalyst immobilized on the crystal by entrapment (deposition from an acrylamide solution), cross-linking, irradiation or pre-coating.

The flow-through piezobiosensor developed by Lasky and Buttry for detecting glucose [169] uses hexokinase entrapped with a poly(acrylamide) matrix placed onto the gold electrode of a 5-MHz AT-cut quartz crystal. The biosensor response is roughly linear for the glucose concentrations typically found in blood (*ca.* 7 mM) and the response time short enough to allow real-time detection. Even though the frequency changes involved are quite reproducible, they are seemingly too large to arise solely from the mass change due to the binding reaction between glucose and hexokinase. One plausible explanation is that the piezoelectric crystal senses changes in the viscoelastic properties of the poly(acrylamide)/enzyme film induced by the binding reaction. Since the frequency change is related to the viscoelastic properties of the medium at the surface, the conformational changes in hexokinase induced by glucose binding may well alter the viscoelastic

properties of the poly(acrylamide) matrix and hence give rise to the large decreases in frequency change observed. Subsequent studies on α -amylase immobilization revealed the sensor to behave similarly [170], so further research is needed in order that the potential of this type of biosensor can be more efficiently exploited.

More recently, Yang and Thompson implemented this type of sensor in FI manifolds, which they consider ideal environments for relating the sensor's hydrodynamic response to the analyte's concentration-time profile produced by the dispersion behaviour of sample zones. Network analysis of the sensor generates multi-dimensional information on the bulk properties of the liquid sample and surface properties at the liquid/solid interface. The relationship between acoustic energy transmission and the interfacial structure, viscosity, density and dielectric constant of the analyte have been thoroughly studied by using this type of assembly [171].

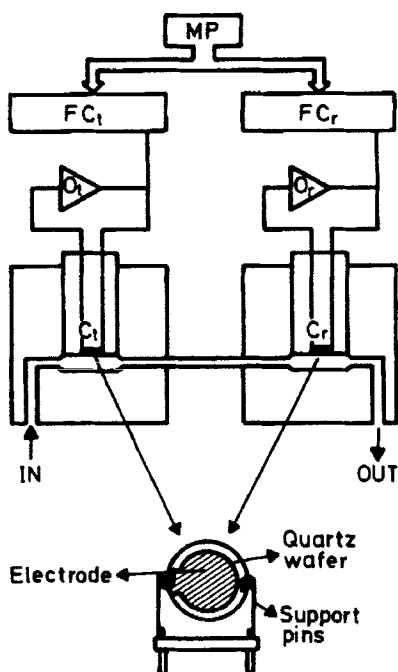


Figure 3.24 — Typical system for piezoelectric crystal detector incorporating reference (C_r) and test (C_t) crystal sensors individually held in oscillating circuits (O_r and O_t , respectively) serviced by separate frequency counters (FC_r and FC_t , respectively) interfaced to a common microprocessor or other readout device. (Reproduced from [167] with permission of the American Chemical Society).

The use of biological catalysts for coating piezoelectric quartz crystals appears to have a promising future as it may help raise their selectivity; also, implementation in flow systems is bound to increase their automatability and throughput. Despite the highly promising results reported so far in this context, the influence of a number of unknown parameters must be established and several shortcomings overcome before any further progress can be made. Luong and Guilbault [172] have outlined a few strategies for action, namely:

(a) Most authors use the Sauerbrey equation in deriving general correlations between the frequency shift and analyte concentration; however, none usually reports any consistency between the frequencies thus obtained and experimental values. This is probably the result of an inability to reproduce monolayer coatings and the added mass on the crystal being distorted during oscillations. For piezoelectric crystals to function properly, both the amount of coating and its distribution on the crystal must be reproducible. The effect of the orientation of coated materials on piezobiosensor performance has yet to be determined. A more complex theory is needed for the thicker, less rigid solid materials. Use of the Z-match theory, which considers the acoustic impedance of a layer attached to the oscillator, makes the quartz crystal microbalance more useful [173]. However, the density and shear modulus of the attached layer must be accurately known.

(b) The problem of cross-reactivity and interference is rather worrisome, particularly with gas and liquid phases. The effects have been significantly reduced, though not fully overcome, by using reference crystals. The greatest limitation of this technique for liquid-phase applications are poor sensitivity and detection limits primarily resulting from non-specific adsorption occurring at both the detector and reference crystal surfaces. Detection limits should be improved by at least three orders of magnitude for these biosensors to acquire clinical significance.

(c) One other critical factor is the molecular size of the analyte to be assayed. These systems appear to work very well with large molecules, whereas attempts at detecting small molecules in the liquid phase have so far met with very little success.

(d) None of the liquid-phase piezobiosensors developed so far can be used for multiple assays since the bound analyte cannot be readily removed from the crystal surface. Thus, the immobilized material gradually breaks away from the surface with repeated use and the crystal sensitivity decreases with time as a result. This may not be significant provided small piezo-

electric quartz crystals can be manufactured in quantities at a very low cost so that disposable transducers are economically feasible.

(e) The amount of humidity and temperature of the gas phase must be carefully controlled since biologically active materials require a certain amount of moisture and feature a very narrow optimal temperature range for proper functioning. These stringent requirements and conditions have made this technique less appealing for some practical applications. Because piezo-electric crystals can vibrate in a liquid phase, a little condensation on the crystal in the gas phase is acceptable. Unfortunately, if the crystal continues to vibrate in the presence of condensation, then the frequency shifts obtained are difficult to define and correlate with the analyte concentration.

(f) A better understanding of the change undergone by the oscillation frequency on immersing a crystal in a solution is needed. So far, the frequency shift is only known to depend primarily on the solution density and viscosity. Also, the resonant frequency is known to decrease with increasing electrolyte concentration and specific conductivity, largely through their proportionality to the solution density. Some authors have reported a linear relationship between the frequency shift and the amount of material adsorbed on the crystal surface, yet others insist that the shift is in no way associated with the classic microgravimetric signal. Both the elasticity (shear modulus) and the viscosity of the medium surrounding crystal electrodes can influence the nature of the frequency available, but some strategies that allow their significance to be assessed have been developed [174].

3.2.2 Non-enzyme sensors

The use of immobilized non-enzyme catalysts for analytical purposes has so far been limited by two deterrent arguments, namely:

- (a) Their selectivity is much lower than that of biological catalysts as they lack the selective or specific active sites of proteins, for example, so they act on —occasionally— very large groups of similar substances and normally require the substrate to be isolated for proper performance.
- (b) As a result of the lack of selectivity, like substances have like effects.

In order to circumvent both shortcomings, achieving adequate catalytic selectivity with a non-biological catalyst entails isolating both the substrate and the catalyst. On the other hand, non-enzyme catalysts are usually much

more inexpensive than are enzymes, so reusability is of much less concern in devising immobilization procedures.

Non-biological catalysts used for constructing flow-through sensors are typically metal oxides and complexes, in addition to some organic compounds. Most of them act via a redox effect, so they are primarily used with electrochemical sensors (amperometric ones in particular).

Flow-through sensors used as parts of composite modified electrodes are built by evenly spreading the catalyst on a suitable support (a conducting polymer matrix) that is compatible with the detector and analyte. The matrix is deposited as a thin film on a supporting electrode surface.

There is a wealth of literature on transport and kinetics in microheterogeneous catalytic systems [175,176], the influence of particle size [177], and complicated situations in which both catalytic microparticles and electron-transfer mediators are dispersed in a polymer matrix [176–179]. The designs and uses of this type of flow-through sensors have been thoroughly reviewed [180,181].

3.2.2.1 Metal oxides and complexes as catalysts in flow-through amperometric sensors

Even though many enzymes and proteins possess functional groups that can be readily oxidized or reduced by chemical redox agents, it is rare for such compounds to undergo easy oxidation or reduction at electrodes. Rather, for reasons ascribed either to their extended three-dimensional structure and the resulting inaccessibility of the electroactive site or their strong adsorption at the electrode surface followed by passivation, most biological macromolecules exhibit such slow electron transfers that no useful currents are generated at ordinary electrodes, not even when applied by rather large overpotentials. The presence of a metal oxide facilitates oxidation of these compounds by substantially lowering the potential for the electrode process. Ruthenium oxides are excellent for this purpose.

A glassy carbon electrode modified by electrodeposition of a film consisting of mixed-valence ruthenium oxide stabilized by cyano cross-links catalyses the oxidation of methionine at 0.92 V vs Ag/AgCl. A glassy carbon sensor was constructed by modifying one such electrode by cyclic voltammetry in a solution consisting of 2 mM RuCl_3 , 2 mM $\text{K}_4\text{Ru}(\text{CN})_6$ and 0.5 M KCl at pH 2. The sensor was accommodated in a flow-cell included in a continuous-flow system. The flow injection amperometric calibration graph thus obtained was linear from 0.6 to 180 μM for a sample volume of 7.5

μL . The sensitivity was $0.3 \text{ nA}/\mu\text{mol}$ at a 0.07-cm^2 electrode and remained constant for over 3 weeks of daily use. The sensor could also be used with carriers typically employed as liquid chromatographic eluents, even though the sensitivity was somewhat lower than that provided by $0.2 \text{ M K}_2\text{SO}_4$ at pH 2, which was seemingly the result of a combined action of the pH and ionic strength. One major constraint to using the sensor in HPLC was that it must be stored in contact with the electrolyte in order to avoid a long reactivation step; more important, baseline stabilization took as long as 1–2 h when the eluent composition was changed. For any given eluent, though, the drift was less than 1 nA/h after the initial pretreatment [182].

Ruthenium dioxide-modified carbon paste electrodes (usually consisting of 40:40:20 graphite/oil/ RuO_2) exhibit a catalytic response to alcohols. This electrocatalytic behaviour was exploited for developing a highly stable and sensitive flow-detection scheme for lower alcohols with a constant-potential operation ($+0.4 \text{ V vs Ag/AgCl}$). The system surpasses other electrochemical detectors for lower alcohols in catalytic stability. Its short-term stability was studied by measuring the flow injection peak current for 20 mM methanol over a 120-min period. The relative standard deviation for the 60 injections performed was 3.4%. Long-term stability was monitored by measuring the steady-state current for 2 mM ethanol over a 13-day period using the same electrode surface. The RuO_2 chemically modified electrode worked as expected throughout the sample batch, with a very slight decrease (*ca.* 6%) in the response and an RSD of 4.0% [183].

Nickel electrodes and glassy carbon electrodes coated with electrochemically deposited films of this metal have catalytic effects on some organic compounds such as carbohydrates arising from the presence of Ni(III) ion formed on application of an appropriate potential. Nickel(III) surfaces act as strong oxidants that react with the carbohydrate by hydrogen atom abstraction to yield a radical. Figure 3.25 shows a tubular electrolytic cell used to exploit this effect. It was constructed by inserting a nickel wire (*ca.* 5-mm long \times 25 or 50 μm OD) into a fused silica tube (50 or 100 μm ID). The effective cell volume thus obtained was less than 30 nL. The system was used to detect sugars such as aldopentoses, aldohexoses, ketohexoses, disaccharides and trisaccharides. A detection limit of 0.1 ng glucose was obtained by using micro FI and HPLC systems at a signal-to-noise ratio of 3 [184]. A nickel-modified glassy carbon electrode was also used to sense the same types of analytes [185].

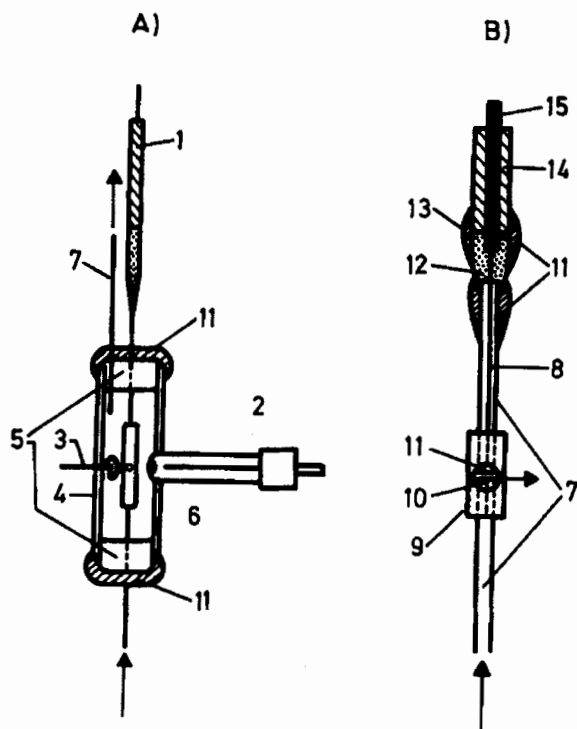


Figure 3.25 — Electrolytic flow-cell of the tubular type. (A) Whole cell. (B) Detail of working micro-electrode: 1 Working electrode; 2 reference electrode (Ag/AgCl); 3 counter-electrode (Pt wire); 4 acrylic tube; 5 rubber cup; 6 electrolyte solution (mobile phase); 7 fused-silica tube (50- or 100- μm ID); 8 Ni wire (diameter 25 or 50 μm , length 5 mm); 9 PTFE tube (0.1-mm ID, 2-mm OD); 10 hole; 11 adhesive resin; 12 glass pipette; 13 silver paste; 14 insulator; 15 electric wire. (Reproduced from [184] with permission of Elsevier Science Publishers).

The effect of fluoride ions on the electrochemical behaviour of a metal zirconium electrode was studied by Pihlar and Cencic in order to develop a sensor for the determination of zirconium ion. Because elemental zirconium is always covered by an oxide layer, the anodic characteristics of a Zr/ZrO_2 electrode are closely related to the composition of the electrolyte in contact with it. These authors found the fluoride concentration and anodic current density to be proportional in hydrochloric and perchloric acid solutions only. In other electrolytes, the fluoride ion-induced dissolution of elemental zirconium led to an increase in the ZrO_2 film thickness and hindered mass transport of fluoride through the oxide layer as a result. The

mechanism of action of the zirconium electrode dissolution at low fluoride concentrations (below 1×10^{-3} M) appears to be different from that at high concentrations, and the dependence of the equilibrium potential on the fluoride concentration is far from Nernstian. A thin-layer cell accommodating an electrode of this type was used for the determination of fluoride in nanogram amounts using a flow manifold [186].

Sensor arrays in which each element is only partially selective towards different analytes are of great interest for practical analytical purposes. The array's response pattern can be coupled to an appropriate chemometric (pattern recognition) approach in order to obtain enhanced chemical information. Most reports on electrode arrays involve potentiometric measurements [187–189]. Several amperometric electrode arrays have been constructed from various electrode materials [190] or permselective coatings [191]. The approach devised by Wang's group involves incorporating various metal oxide catalysts into an array of carbon paste electrodes. Because each modifier exhibits a different electrocatalytic behaviour to a given analyte, the information content and hence the selectivity are greatly improved. The overall assembly used for this purpose is depicted in Fig. 3.26. It consists of a flow injection manifold including a four-electrode array and a high-volume wall-jet detector. Four cavities (3 mm in diameter) are equally spaced at the bottom of a 12-mm diameter Plexiglas cylinder (3 mm from the centre) and filled with modified carbon paste prepared by thoroughly hand-mixing the desired amount of metal oxide with Acheson 38 graphite powder and mineral oil in a 25:45:30 proportion by weight. The electrode surfaces are smoothed on a piece of weighing paper. By coupling the unique sensor array response patterns obtained to various statistical regression techniques, individual carbohydrates or aminoacids can be determined in different sample mixtures. For binary and ternary mixtures, the partial least-squares calibration method yielded an average relative prediction error of 2.3%. Further improvements in the information content can be achieved by applying different potentials at the individual channels. The resulting array response, consisting of full hydrodynamic voltammograms at each modified electrode, offers unique qualitative information based on the potential dependence of the catalytic response [192].

A thin film of manganese oxides deposited over a glassy carbon electrode dramatically lowers the overpotential for oxidation of various hydrazines and hydrogen peroxide, thereby facilitating their amperometric detection in flow systems. Sensors based on this principle are highly sensitive and provide

detection limits at the femto- and picomole levels, and relative standard deviations less than 1.3% [193].

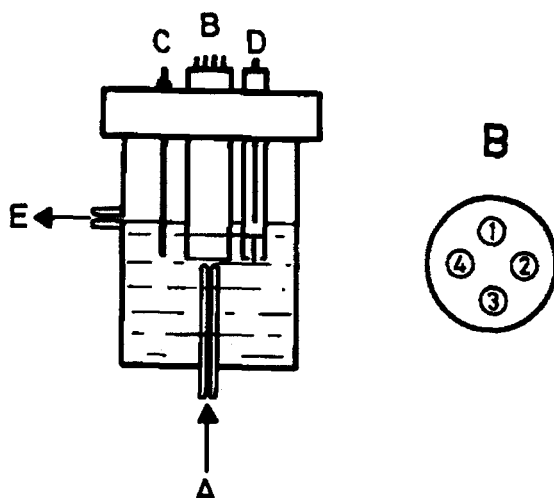


Figure 3.26 — Schematic view of large volume wall-jet detector: (A) inlet; (B) electrode array; (C) counter-electrode; (D) reference electrode; (E) outlet. Also shown (right): bottom view of the electrode array. (Reproduced from [191] with permission of the American Chemical Society).

The electrooxidation of hydrazine is also catalysed by metal compounds other than oxides, as shown by Hou *et al.* using an FI manifold. They employed a cobalt tetraphenylporphyrin-modified glassy carbon electrode to oxidize hydrazine at +0.5 V vs Ag/AgCl and pH 2.5, and achieved a detection limit of 0.1 ng [194].

The electroreduction of some typically inorganic compounds such as nitrogen oxides is catalysed by the presence of polymeric osmium complexes such as $[\text{Os}(\text{bipy})_2(\text{PVP})_{20}\text{Cl}]\text{Cl}$, where bipy denotes 2,2'-bipyridyl and PVP poly(4-vinylpyridine). This polymer modifies the reduction kinetics of nitrite relative to the reaction at a bare carbon electrode, and provides calibration graphs of slope 0.197 nA with detection limits of 0.1 $\mu\text{g/mL}$ and excellent short-term reproducibility (RSD = 2.15% for $n = 20$). The sensor performance was found to scarcely change after 3 weeks of use in a flow system into which 240 standards and 30 meat extracts were injected [195].

Mottola *et al.* developed two types of modified electrodes containing reversible Fe(II)/Fe(III) sites for the determination of nitrogen oxides. One was made by directly mixing carbon paste with *tris*[4,7-diphenyl-1,10-phenanthroline]iron(II) perchlorate while the other consisted of a glassy carbon surface modified by oxidative electropolymerization of *tris*[5-amino-1,10-phenanthroline]iron(II) perchlorate. Both were used in flow systems. Continuous "bathing" of the sensor surface with supporting electrolyte ensured the presence of an unbroken film of ions to support the electrical migration and satisfy the electroneutrality requirement. The polymer coated sensor compared favourably with the chemically modified carbon paste electrode, and exhibited excellent resistance to poisoning and a competitive detection limit (*ca.* 2 ng/mL) at +1.0 V vs Ag/AgCl, in addition to good selectivity towards NO₂ when used in a thin-layer cell inserted in a flow manifold that allowed up to 120 samples/h to be processed. The typical concentration range amenable to determination was 2–25 ng/mL, but depended on the polymer film thickness [196].

3.2.2.2 Organic compounds as mediators in flow-through amperometric sensors

One alternative approach to obtaining an electrochemical response to enzymes and proteins (and, in general, organic or even inorganic species) involves using low molecular weight mediator titrants [197,198], some of which exhibit an ideal reversible electrode behaviour. These species are added to the sample solution and serve as electron-transfer mediators by effecting the chemical oxidation or reduction of the analyte away from the electrode surface. The mediator effects the desired redox conversion of the analyte at a chemical step following its own electrolysis at its characteristic potential. In the light of developments in chemically modified electrodes (CMEs), one logical and attractive extension of the mediator titrant approach is construction of CMEs using proven solution phase mediators as the electrode-modifying agents. Some such CMEs have indeed been designed and reported that include derived viologen-modified silicon and glassy carbon electrodes [199,200], and bipyridyl promoter-adsorbed gold and platinum electrodes [201–205]. Electropolymerization of viologens onto gold [206–208] yielded electrodes that exhibited *quasi*-reversible activity towards cytochrome c, myoglobin and ferredoxin. A Methylene Blue chemically modified electrode was constructed and studied spectroelectrochemically as a mediator for the determination of myoglobin in a flow-through system

[209]. Electrodeposition of Toluidine Blue onto a glassy carbon substrate using cyclic voltammetry was employed to construct a CME that effected electrocatalytic reduction of myoglobin and hemoglobin. Use of this sensor in a flow injection manifold at a constant applied potential of -0.30 V vs SCE provided detection limits of 20 and 50 ng (1.2 and 0.78 pmol) injected myoglobin and hemoglobin, respectively, with a dynamic linear concentration range over 2 orders of magnitude. Several redox proteins in addition to myoglobin and hemoglobin were examined by using the FI manifold and a CME including hemoprotein-containing cytochrome c and such non-hemoproteins as glucose oxidase, alcohol dehydrogenase and bovine serum albumin. The hemoproteins showed evidence of catalytic response at the CME, whereas non-hemoproteins did not. One potential application for this CME would be detection of myoglobin in human blood. Since, however, the myoglobin concentration level in normal human blood is 10–100 ng/mL, the detection limit of the sensor in its present form would have to be improved [210].

Re-oxidation of the reduced form of NAD^+ for detection of dehydrogenase-catalysed reactions has been improved by several authors by modifying classic graphite electrodes with various organic compounds that exhibit catalytic action on the oxidation of a co-enzyme. Thus, a graphite rod electrode can be simply modified by dipping it in a solution of 7-dimethylamino-1,2-benzophenoxazinium salt (Meldola Blue). By mounting one such sensor in a flow-cell inserted in a flow injection manifold, sample volumes of 50 μL can be used to obtain linear calibration ranges from 1 μM to 10 mM with an RSD of 0.2–0.6%, which allows application to the determination of substrates involved in dehydrogenase-catalysed reactions in biological fluids [211]. The presence of phenoxazinium ions in these sensors has a similar effect to that of the previous dye. Insertion of a separation unit in the continuous-flow system used to implement this type of sensor results in improved response to complex samples (*e.g.* enzymatic determinations in biological fluids) [212].

The organic conductor properties of tetrathiafulvalenetetracyanoquinodimethane (TTF–TCNQ) as a material for constructing electrodes, *viz.* its catalytic response and resistance to passivation, are of special interest for the determination of biological compounds, which usually have slow electrode kinetics and a low sensitivity, and tend to foul electrode surfaces. The response of a TTF–TCNQ microarray sensor inserted in a flow system for

the electrochemical determination of straightforward biomolecules has so far provided very promising results [123].

In the presence of Nafion, the ion-exchange mechanism accompanying the redox reactions of polyaniline allows a polyaniline–Nafion composite electrode to act as an electrochemical sensor for cations. This allowed Sung and Huang to construct a flow-through sensor for the determination of alkali and alkaline-earth metal ions by using a simple procedure involving application of diamond paste and alumina over the surface of a previously polished platinum disc electrode, followed by a small film of Nafion dissolved in 9:1 ethanol/water mixture and air-drying to evaporate the solvent. The electrode was then immersed in a stirred solution containing 0.5 M Na_2SO_4 , 0.1 M H_2SO_4 and 0.1 M aniline and a polyaniline film was deposited by sweeping the potential from 0.00 to +1.0 V and back to –0.20 V *vs* Ag/AgCl for three full cycles at a sweep rate of 20 mV/s. The resulting sensor, used in FI manifolds and ion chromatography systems, provided detection limits of *ca.* 2×10^{-7} M for the metals assayed [214].

As shown by Wang and Lin, polyaniline-modified flow-through carbon sensors can also be used for determining anions such as iodide, bromide, thiocyanate and thiosulphate with detection limits of 1, 5, 10 and 10 mg/mL, respectively. For this purpose, a thin film of polyaniline (PAn) was formed over a conventional carbon electrode that is subsequently placed in a continuous (flow injection or ion chromatography) system. The sensor obtained by electropolymerization of aniline at a slow potential cycling (20 $\mu\text{V/s}$) in 1.0 M sulphuric acid was highly stable. The same CME surface could be used for cyclic voltammetry (CV) and FI experiments for 2 weeks or longer with no evidence of chemical or mechanical deterioration. The CME's cyclic voltammetric response to various ions was also maintained at the same level no matter how often the electrode was exposed to other electrolytes during intervening CV scans. However, achieving a constant analytical response for a series of anions in FI systems required some provision to be made for discharge of the anions accumulated in the course of repeated exposures. For a series of 20- μL injections of 1.0×10^{-2} M SO_4^{2-} at +0.06 V *vs* SCE, the oxidation peak currents measured in boric acid fell to only 50% of the initial response after 10 such exposures. However, the decrease in the response was much less severe at lower anion concentrations and for a series of 20- μL injections of 1.0×10^{-4} M SO_4^{2-} (the response was 90% of the initial level after 10 exposures). A discharge procedure previously developed for polypyrrole was used to determine high anion con-

centrations. The CME could be briefly exposed to a reducing potential of *ca.* -0.2 V between successive anion exposures. In this way, the polyaniline film was cycled through its fully reduced state with the accompanying discharge of any anions that had been incorporated as a result of polymer oxidation. One of the most salient features of this electrode, which should be the subject of deeper study, is possibly the selectivity obtained by varying the composition of the electrolyte solution into which the sample is injected [215].

3.3 FLOW-THROUGH IMMUNOSENSORS

Immunosensors exploit the high selectivity provided by the molecular recognition of antibodies. Because of significant differences in their affinity constants, antibodies may endow immunosensors with increased sensitivity relative to enzyme sensors. In addition, antibodies may be obtained in principle against an unlimited number of determinands. Immunosensors are thus characterized by high selectivity, sensitivity and flexibility. These desirable features for a sensor have propitiated extensive research and development on immunosensors in the last two decades —particularly in recent years, as the likely result of both the strong demand for immunoassays and remarkable advances in immunochemical technology. It should be noted that such technologies as optoelectronics have also reached immunosensor development.

Immunosensors can be classified into two broad categories: non-labelled and labelled. Non-labelled immunosensors are designed in such a way that the immunocomplex (*i.e.* the antigen–antibody complex) is directly determined by measuring the physical changes induced by the complex formation. In contrast, labelled immunosensors include a sensitively detectable label, so the immunocomplex is determined by measuring the label.

Non-labelled immunosensors rely on various principles (Fig. 3.27.A). Either the antibody or the antigen is immobilized on the solid matrix to form a sensing device. The solid matrix should be sensitive enough at the surface to detect immunocomplex formation. Electrode, membrane, piezoelectric and optically active surfaces may in principle be used to construct non-labelled immunosensors. The antigen or antibody to be determined is dissolved in a solution and reacted with the complementary matrix-bound antibody or antigen to form an immunocomplex that alters the physical (*e.g.* the electrode potential or intrinsic piezofrequency) or optical properties of the

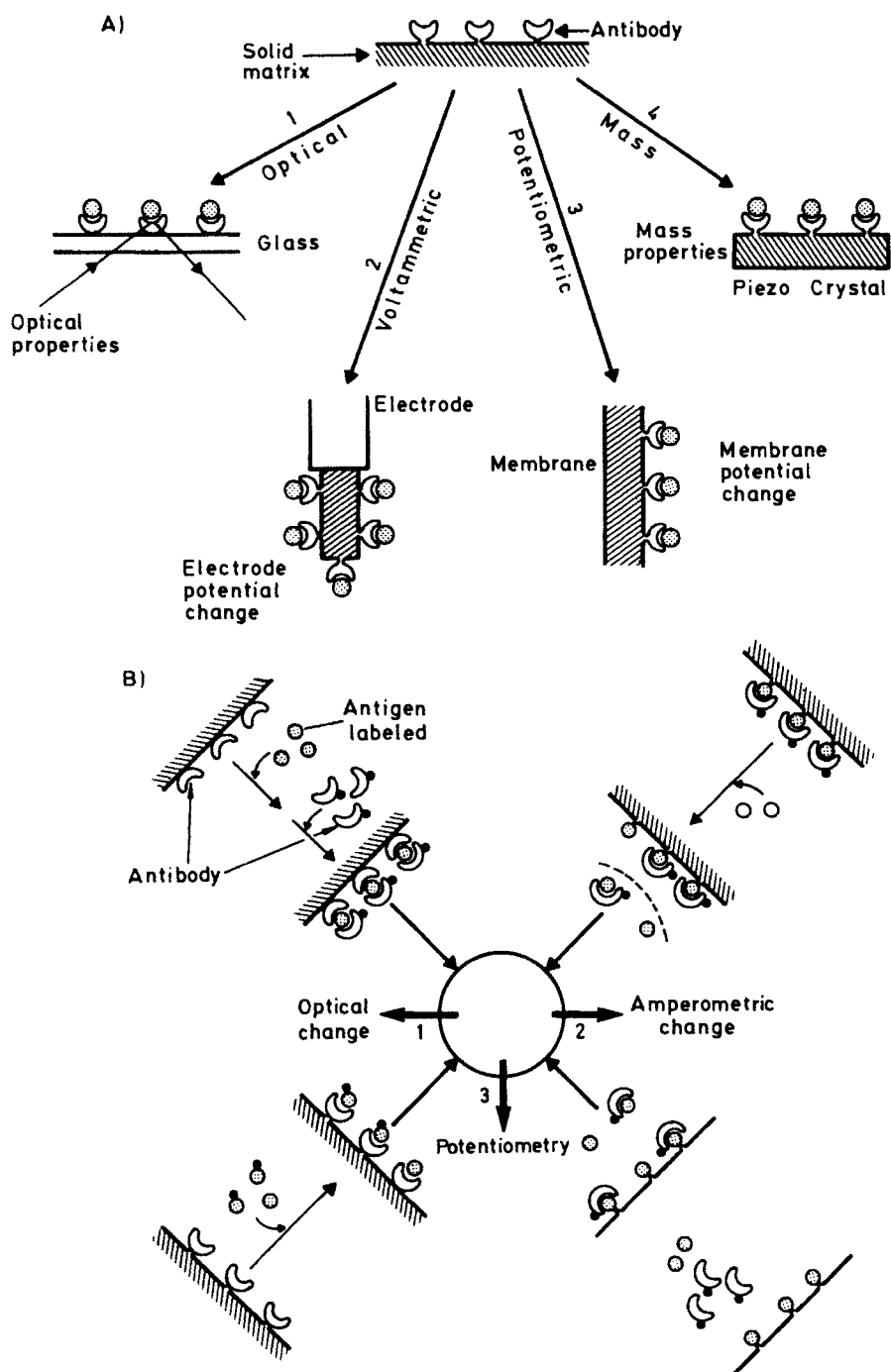


Figure 3.27 — Principles of non-labelled (A) and labelled (B) immunoassay.

surface. While most non-labelled immunosensors provide high enough selectivity, there is still the problem of non-specific adsorption onto the matrix-bound antibody surface.

Much research has gone into raising the sensitivity and selectivity of immunosensors to the desired levels. Several labels have proved to ensure a high sensitivity, yet radioisotopic labels have essentially been avoided. Non-isotopic labels for immunosensors include various enzymes, catalysts, fluorophores, electrochemically active molecules and liposomes. Labelled immunosensors are basically designed so that immunochemical complexation takes place on the surface of the sensor matrix. There are several variants of the procedure used to form an immunocomplex on the matrix. In the final step, however, the label should always be incorporated into the immunocomplex for determination, as shown in Fig. 3.27.B.

Because these sensors are not so varied as those described in Section 3.2.1, they are dealt with in fewer, more general sub-sections. On the other hand, electroluminescence sensors, included in none of the previous sections, are discussed after electrochemical sensors.

3.3.1 Optical flow-through immunosensors

Fibre-optic immunosensors (FOIS) are the most desirable of optical sensors as they can in principle be used for both batch and continuous-flow measurements. However, the practical need for a pre-incubation period—which can be avoided by using an appropriate flow configuration—and the sluggish response of many of these sensors make them unsuitable for use in continuous systems. The intensive research into homogeneous immunosensors has so far materialized in comparatively few papers. Figure 3.28 shows some of the more common ways of attaching an antibody to the tip of an optical fibre. The first was devised to develop a sensor for polynuclear aromatic hydrocarbons (PNAs) based on their native fluorescence [216]; however, its poor sensitivity (probably resulting from both the small amount of antibody that was immobilized and denaturation on bonding to the fibre) and the long incubation required (60 min) made it inappropriate for flow applications. Subsequent research on the sensor has provided promising results, though [217].

Integrated optical immunosensors. A flow-cell containing an affinity reagent can be flexible enough for implementation of all the steps involved in an immunoassay provided it is used in a flexible flow injection manifold that can be adapted as required.

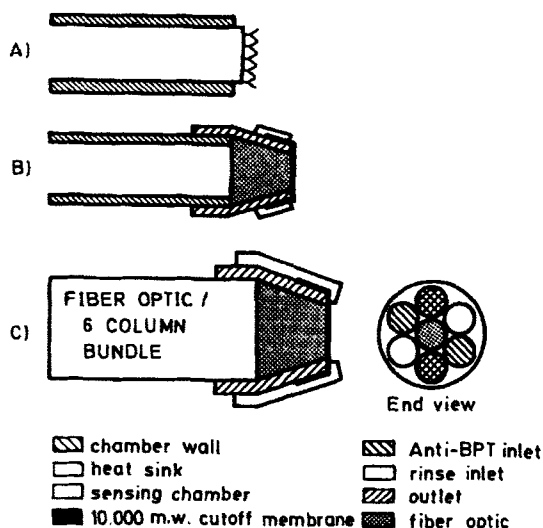


Figure 3.28 — Sensing terminal for antibody-based FOCS: (A) direct covalent attachment of immunochemical, (B) membrane entrapment of immunochemical, and (C) a regenerable immunosensor. (Reproduced from [216] with permission of the American Chemical Society).

Figure 3.29.A shows a flow-cell of 20 μL inner volume used to hold immobilized anti-mouse IgG bound to a rigid beaded support (activated Pierce trisacryl GF-2000). The cell was used to develop a two-site immunoassay for mouse IgG by consecutive injection of the sample, acridinium ester-labelled antibody and alkaline hydrogen peroxide to initiate the chemiluminescence, which started the reaction sequence shown in Fig. 3.29.B. Regenerating the sensor entailed subsequent injection of an acid solution, which resulted in a determination time of *ca.* 12 min (this varied as a function of the flow-rate used, which also determined the detection limit achieved, *viz.* 50 amol for an overall analysis time of 18 min) [218]. The sensor was used for at least one week with an inter-assay RSD of 5.9%. Attempts at automating the hydrodynamic system for use in routine analyses are currently under way.

The non-competitive FI immunoassay for haptens recently reported by Gunaratna and Wilson [219] offers rather an interesting means for developing flow-through sensors for this type of compound.

Wilson *et al.* developed a flow-through immunosensor using an immunosorbent membrane holding an antigen (bovine IgG) for the determination of

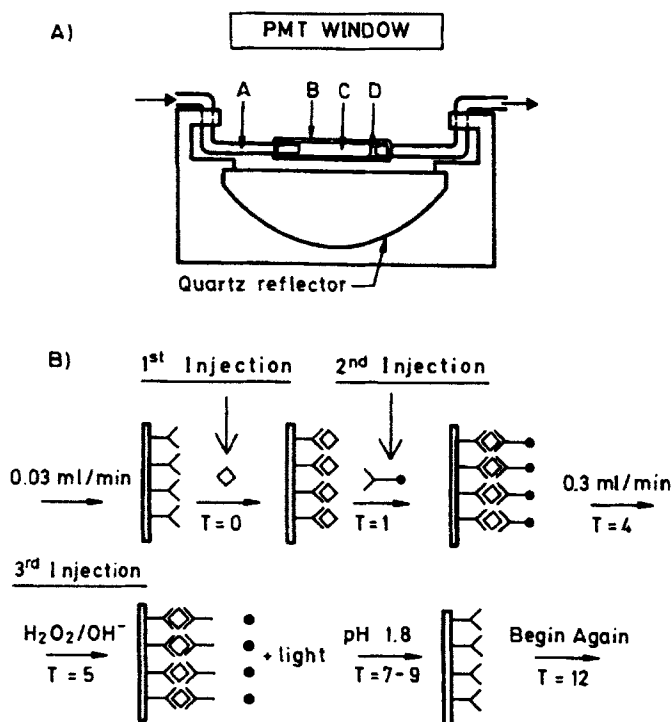


Figure 3.29 — (A) Immunosensor scheme: A Cell inlet tubing; B transparent PTFE tube (1.6-mm ID \times 3-mm OD; C immunosorbent; D frit. (B) Outline of flow-injection immunoassay procedure. The assay buffer is phosphate buffered saline (PBS) at pH 7, and flow-rates and times (min) are given in the figure. Immobilized anti-mouse IgG; modified sample (mouse IgG) injected at $T = 0$; change of the flow-rate and buffer at $T = 4$; injection of hydrogen peroxide in a basic medium at $T = 5$; then, emission monitoring and regeneration step; acridinium ester-labelled antibody (emitter = N-methylacridine). (Reproduced from [218] with permission of Elsevier Science Publishers).

mouse anti-bovine IgG by sandwich ELISA immunoassay in a continuous system including a chemiluminescence detector. Figure 3.30.B depicts the thin-layer flow-cell immunosensor. It consisted of a $0.5 \times 3 \text{ cm}^2$ piece of bovine IgG-coupled membrane and a Teflon spacer sandwiched between two transparent Plexiglas plates. The spacer created a 0.15-cm wide, 0.01-cm thick and 2.5-cm long channel of *ca.* $5 \mu\text{L}$ volume. The membrane surface area exposed to the flowing stream was *ca.* 0.4 cm^2 . The two Plexiglas plates were held together by four screws and the assembly was directly mounted in front of a photomultiplier window. The membrane was made from

polyvinylidene difluoride, which exhibited very low non-specific adsorption. It was hydrophilic and porous, and was activated chemically for coupling with a primary amine group of protein in an aqueous solution. The analytical performance of the membrane-based biosensor was assessed by means of the sequential injection illustrated in Fig. 3.30.A at a flow-rate of 0.1 mL/min. Each assay cycle consisted of four injections, namely: (1) an unknown or standard mouse anti-bovine solution ($t = 0$); (2) goat anti-mouse-HRP ($t = 2$ min); (3) buffer of pH 7.4 ($t = 4$ min); and (4) the enzyme substrate and luminol reagents ($t = 6$ min). The 2-min interval between injections was imposed by the particular autosampler used (one with a faster cycle time could have resulted in sampling intervals perhaps as short as 15–30 s). The amount of anti-mouse-HRP injected was 1.5 pmol (*i.e.* more than one order of magnitude larger than that of mouse anti-bovine injected) in order to ensure that all the retained mouse anti-bovine antibody was sandwiched. Buffer injections served two purposes, *viz.* (a) to prevent potential carry-over of the enzyme conjugate in the injector sample loop and (b) to provide a long enough washing time for the non-specifically adsorbed labelled antibody. The reproducibility of the CL detection and stability of the sandwich complex were demonstrated by re-injections of the enzyme substrate luminol reagents. Triplicate injection cycles involving the same analyte solution yielded peak areas with an RSD of 5%. A calibration graph was run by plotting peak area increments against the concentration (in femtomol) of mouse anti-bovine antibody injected each time. The calibration curve thus obtained was linear, with a slope of 0.028, an intercept of 0.0 and a correlation coefficient of 0.99. However, washing with buffer of pH 2.2 to regenerate the immunosorbent was wound to reduce the membrane capacity as the likely

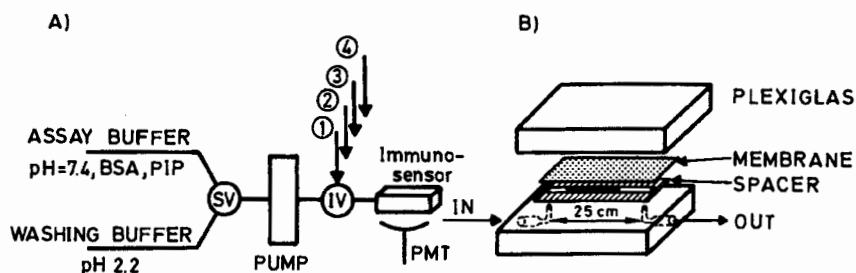


Figure 3.30 — (A) Flow-injection manifold for implementation of immunosensors. (B) Scheme of a thin-layer flow-through immunosensor. (Reproduced from [220] with permission of the American Chemical Society).

result of denaturation or loss of immobilized antibody, which was adsorbed rather than covalently attached. The output signal was found to decrease by *ca.* 20% after three injections, so the membrane was replaced with a fresh one with that frequency. Replacement was quite simple and expeditious, usually taking only about 2 min. The detection limit achieved with a fresh membrane was *ca.* 1 fmol at a signal-to-noise ratio of 3. Notwithstanding its pitfalls, the sensor has a promising future for solid-phase assays since the membrane immunosorbent configuration allows analysis of samples that are turbid or contain particulate matter, which would otherwise clog a packed reactor [220].

3.3.2 Electrochemical flow-through immunosensors

Electrochemical immunosensors are the most numerous of immunosensors. Their suitability for use in flow systems is limited by the two factors commented on above in dealing with fibre optic-based sensors, *viz.* the need for an incubation period and exceedingly long response times.

3.3.2.1 Amperometric immunosensors

The amperometric immunosensors reported so far rely on various methodological principles including use of a Clark electrode for detecting oxygen formation or depletion, an electrochemically active product yielded in an enzyme reaction or an antigen labelled with an electroactive species.

The sensor developed by Aizawa *et al.* in 1979 for monitoring human chorionic gonadotropin (hCG) using catalase-labelled hCG can readily be adapted for use in a flow injection system in order to implement the different steps involved in the analyses. The sensor consists of an antibody immobilized on a pre-cast membrane that is placed on the Teflon membrane of an oxygen electrode. Both labelled and non-labelled hCG are allowed to compete for the antibody in the membrane, which is washed to remove bound from free hCG, and the electrode is exposed to an H_2O_2 solution. In the presence of catalase, the hydrogen peroxide disproportionates to yield oxygen and water. The sensor is used to monitor the rate of increase in oxygen tension (it can monitor between 0.02 and 100 IU hCG/mL based on a calibration plot). Unfortunately, the assay is prone to cross-reactivity with luteinizing hormone. However, the availability of good monoclonal antibodies against the *a* and *b* subunits of hCG and the use of a sandwich-type ELISA involving not labelled hCG, but a labelled second antibody, could be the basis for development of an improved biosensor for hCG. The same

methodological principle was used to develop an immunosensor for the determination of theophylline [222].

An additional use of the Clark electrode in connection with immunosensors is for measuring oxygen consumption, as in the enzyme-linked immunoassay for factor VIII-related antigen using a glucose oxidase electrode modified for monitoring the glucose produced by an antibody labelled with alkaline phosphatase [223]. The assay is very complicated and uses rather expensive reagents, though.

Schramm *et al.* developed several dual-antibody systems for constructing biosensors based on competitive immunoassay methodologies. The two antibodies were immobilized at separate locations: one (Ab-1) was used to recognize the analyte and analyte-enzyme conjugate, while the other (Ab-2) was employed to recognize the enzyme component in the conjugate. This dual antibody system provides two signals originating from the analyte-enzyme conjugate bound to Ab-1 and Ab-2 in response to different analyte concentrations in the samples, and enables implementation of a competitive immunoassay without separation of bound and unbound analyte-enzyme conjugate by the user. Of the three variants proposed by the authors, two were irreversible, whereas the third, of the reversible type, was suitable for continuous monitoring of analytes in flow systems. Preliminary experiments with progesterone-HRP as the analyte-enzyme conjugate involving colorimetric measurements of enzyme activity entailed destroying the analytical cell and did not allow the signals at the two antibody sites to be monitored. By using electrochemical sensors such as that shown in Fig. 3.31.A [224], the hydrogen peroxide formed by the glucose oxidase in the analyte-enzyme conjugate was measured amperometrically. The H_2O_2 released into the bulk solution from one electrode was prevented from reaching the other by a mesh containing immobilized catalase. The sensor's time response was rate-limited by such factors as (a) the permeation rate of the antigen through the semi-permeable membrane, (b) the binding constants of the two antibodies to their respective antigens, and (c) the half-life of the enzyme in the sensor compartment if enzymes were used as signal generators [225].

Several amperometric immunosensors have been developed for monoclonal antibodies (IgG), α -interferon and the pesticide 2,4-dichlorophenoxyacetic acid (2,4-D) by using a flow-cell with the catching antibody covalently bound to a cellulose acetate or activated nylon membrane as shown in Fig. 3.31.B. With multiepitope antigens (*e.g.* a protein), after the antigen is bound and washed, a second enzyme-labelled antibody is used to form a sandwich

structure. With haptens (*e.g.* pesticides), the hapten molecule is conjugated with an enzyme and the labelled and free antigens compete for the catching antibodies. Glucose oxidase (GOD) and alkaline phosphatase (AP) as marker enzymes yield signals at 0.6 and 0.1 V with glucose and 4-aminophenyl-phosphate, respectively, which correspond to the bound enzyme; such a sensitivity is more than adequate for practical bioreactor monitoring. The sensitivity for pesticides, however, should be dramatically increased for practical purposes [226].

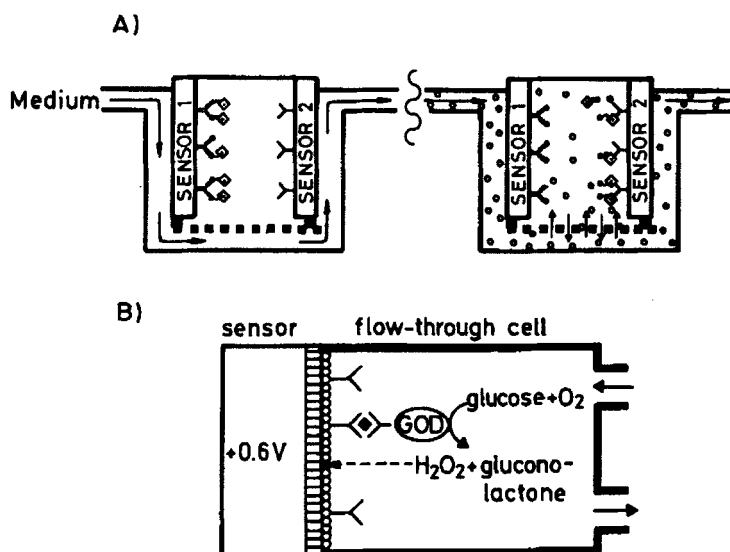


Figure 3.31 — (A) Continuous monitoring of analytes in a fermentation medium. In the absence of analyte, the analyte enzyme conjugate binds predominantly to sensor 1 (left). At high analyte concentrations in the medium, the signal shifts to sensor 2 (right). (B) Principle of the membrane immunosensor. (Reproduced from [225] and [226] with permission of VCH Publishers).

Some electrochemically active substances that can generate photons on an electrode surface are suitable labels for homogeneous immunoassays. A labelled antigen exhibits an electrochemical reactivity and produces luminescence, but when it is immunochemically complexed, the labelled antigen loses its electrochemiluminescent properties. One optical immunosensor for homogeneous immunoassays was assembled by spattering platinum on the end surface of an optical fibre. Spattered platinum maintains optical transparency and functions as an electrode. An optical electrode efficiently

collects photons produced on the surface of the transparent electrode. Luminol as a label offers excellent features for designing a homogeneous immunosensor and produces chemiluminescence by two different types of electrochemical excitation. One is based on a two-step electrochemical excitation: cathodic excitation followed by anodic excitation. The former produces hydrogen peroxide that causes anodically generated luminol radical to emit photons. The latter is a single-step electrochemical excitation in the presence of hydrogen peroxide. Luminol is simply oxidized by anodic excitation to generate radicals, followed by emission of photons. The two-step electrochemical excitation provides a very high sensitivity and a detection limit of 10^{-13} M luminol. Homogeneous immunoassays with IgG as the model antigen, labelled with luminol, were carried out in this way. Similarly to free luminol, labelled IgG produces electrochemical luminescence by anodic oxidation in the presence of hydrogen peroxide. Electroluminescence sharply decreases through immunocomplexation with anti-IgG antibody (addition of 10^{-13} g/L antibody was found to result in significantly suppressed luminescence, with the lower detection limit falling in the range 10^{-13} g/L antibody) [227].

3.3.2.2 Potentiometric immunosensors

There are three basic types of potentiometric immunosensors. One is used for the determination of transmembrane potentials across an antibody (or antigen) membrane that specifically binds the corresponding antigen (antibody) in solution. Concentrations of either the target antigen or antibody can be determined by measuring changes at the membrane surface. Another type relies on measurements of electrode potentials. The surface of an electrode is modified by using an antibody or antigen capable of binding specifically to the corresponding antigen or antibody. Formation of an immunocomplex causes the electrode potential to change, primarily as a result of a change in the surface charge related to the concentration of dissolved analyte. The third type of potentiometric immunosensor is used for measuring surface potentials at the gate of a field effect transistor covered with a thin antibody binding membrane. The surface potential of the FET gate may vary with the antigen concentration in solution.

Aizawa *et al.* developed immunosensors of the first type for syphilis and blood typing [228–230] based on measurements of the transmembrane potential across an immunoresponsive membrane. The potentiometric immunosensor using an antibody against human chorionic gonadotropin (hCG)

hormone covalently bound to an electrode surface, which responds to dissolved hCG, is of the second type [231]. Notwithstanding their operational simplicity, these potentiometric immunosensors lack sensitivity.

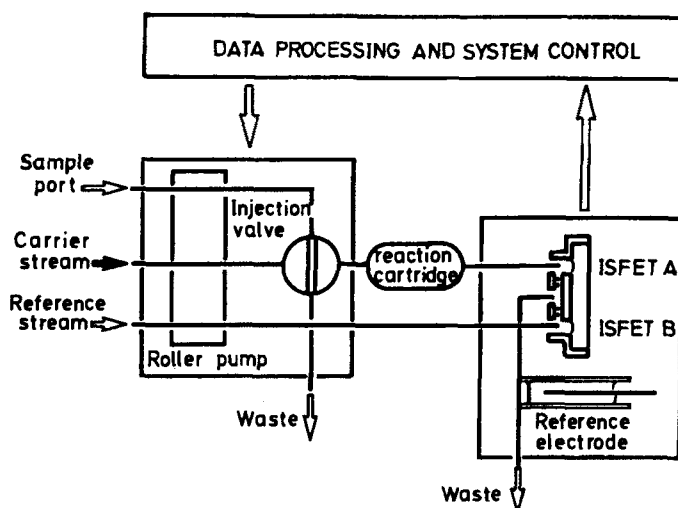


Figure 3.32 — ISFT-ELISA immunosensor for HIV-serology. (Reproduced from [232] with permission of VCH Publishers).

An ISFET-ELISA system for HIV serology was included in a flow injection manifold to develop the flow-through immunosensor system depicted in Fig. 3.32. In accordance with the conventional ELISA principle, immunoprecipitate formation is recognized by a specific urease-labelled antibody. The enzyme urease yields a pH shift in the presence of urea which can be detected by means of an ISFET. Formation of the immunocomplexes and the enzyme-catalysed reaction take place in separate cartridges packed with suitable supporting materials. The chief advantages of this set-up are the high sensitivity and flexibility of the ensuing analytical method. A comparative study involving various cartridge geometries and supporting materials (*e.g.* packed beds, perfused membranes, flow-by configurations) was performed. Various immobilization techniques including covalent bonding and adsorptive binding were assayed on the different supporting materials. In order to increase the sensitivity, the flow injection system was optimized in terms of dead space and flow geometry. The ISFET was accommodated in a two-channel flow-through cell with an overall dead

volume of *ca.* 7 μL . By using an appropriate encapsulation technique, the liquid head space above the ISFET sensor was reduced to *ca.* 1 μL . Linear calibration curves over the range 10^{-9} – 10^{-4} g/L were obtained for HIV-antibodies by using immobilized p24 antigens and a precoated nitrocellulose membrane [232].

3.3.2.3 Capacitance immunosensors

The concept of capacitive affinity sensor is based on the principle that the capacitance of electrolytic capacitors depends on the thickness and dielectric properties of a dielectric layer on the surface of a metal plate. Hence, an electrolytic capacitor allows detection of material bound to a plate, which in principle allows one to detect any analyte binding to a specific ligand immobilized on the insulating layer of a plate. By way of example, Fig. 3.33.A illustrates the binding of an antigen to an immobilized antibody [233]. The space between the immobilized protein molecules is filled with electrolyte (there is a conductor connected in parallel with the capacitor, as illustrated by the model in Fig. 3.33.B). A measuring method or instrument that can distinguish between capacitance and conductance changes on the basis of their different phase characteristics is required. The capacitance of antibodies depends on several properties that cannot be used as design parameters. The only one such parameter that can be optimized is the thickness of the insulating layer; for maximum capacitance, the layer should be of minimal thickness, non-porous and insoluble.

Figure 3.33.C depicts the tantalum capacitance flow-through cell for real-time monitoring of immunochemical interactions reported by Gebber *et al.* Two sensor strips consisting of tantalum substrate (1) onto which tantalum oxide (2) has been grown, are separated by a Teflon spacer (3). The spacer has a special shape (b in Fig. 3.33.C) in order to prevent entrapment of air bubbles in the flow-through cell. The sample fluid flows through the spacer from the broader to the narrower end. During passage through the cell chamber, the flow velocity increases, thus creating a pressure difference between the top and bottom of the air bubbler. Therefore, as a result of the vertical placement of the cell, bubbles are forced to exit chamber. The sensor parts are clamped together by two Teflon plates (4). The tantalum electrodes are connected by using the four-electrode technique [223]. Finally, the sensor is shielded in a metal box (5). The antibody or antigen used is immobilized on the electrodes and binding of the analyte concerned results in an electrical

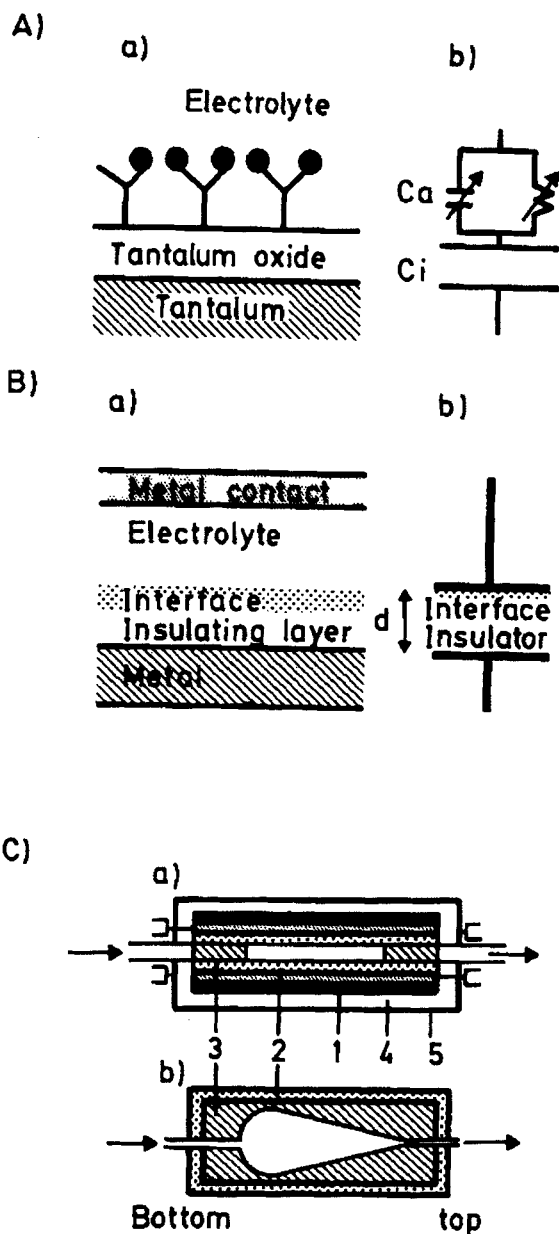


Figure 3.33 — (A) Analyte binding to antibodies immobilized onto a sensor surface (a) and electric model used to represent it (b). (B) Illustration of the concept of electrolytic capacitor: (a) schematic and (b) electric description. (C) Capacitance-based immunosensor: (a) vertical section; (b) horizontal section; 1 tantalum foil; 2 tantalum oxide; 3 Teflon spacer; 4 Teflon plates; 5 metal box. (Reproduced from [234] with permission of the American Chemical Society).

capacitance change. With mouse IgG as the ligand, real-time monitoring of nanogram-per-millilitre concentrations of anti-mouse IgG is possible [234].

3.3.3 Calorimetric flow-through immunosensors

In addition to the above-described applications, enzyme thermistors are potentially useful in such a rapidly growing area as immunoassays. The name "thermometric enzyme-linked immunosorbent assay" (TELISA) has been coined to designate the technique used to implement enzyme thermistor-based sensors used in this type of assay [235]. In principle, the column holding the enzyme thermistor is filled with immunosorbent (*e.g.* an antibody immobilized on Sepharose CL-4B). The antigen to be determined and the enzyme-labelled antigen are inserted into the flow, the residual amount of enzyme-bound antigen that remains bound to the column after that being a function of the antigen content. The less antigen is present in the sample, the more enzyme-labelled antigen will be found in the column and thus the more heat will evolve on subsequent introduction of substrate into the flow stream. After the determination, the immunosorbent is readily regenerated by washing with glycine at a low pH and a measuring cycle takes only 10–15 min to complete.

An immunosensor based on this principle was used for the determination and monitoring production and release of human proinsulin by genetically engineered *Escherichia coli* cells [236]. Anti-insulin antibodies were affinity purified against beef insulin. The fraction eluted with 0.2 M glycine hydrochloride at pH 2.2 was coupled to Sepharose 4B activated with tressyl chloride. A beef insulin–peroxidase conjugate was made and the fraction with a weight ratio of insulin to peroxidase of 1:2 was isolated by chromatography. In this form of TELISA, which is a competitive assay, the unlabelled antigen in the sample (or standard) is mixed with a fixed amount of enzyme-labelled antigen and the mixture is then applied to the immunosorbent column mounted in the enzyme thermistor apparatus. As the amount of unlabelled antigen in the sample increases, that of enzyme-labelled antigen bound to the column decreases. The amount of peroxidase bound to the column is determined by injecting a substrate pulse containing 2 mM H₂O₂, 14 mM phenol and 0.8 mM 4-aminoantipyrine. Finally, bound antigen is removed from the immunosorbent by washing with 0.2 M glycine hydrochloride at pH 2.2, thereby making the column ready for the next assay. The automated assay can be controlled via a programmable unit that actuates the values for different eluents and substrates, as well as the sample changer.

Readings can be obtained within 7 min after a sample is introduced, and a single assay cycle is completed in 13 min. Insulin concentrations in the range 0.1–50 $\mu\text{g/mL}$ can thus be determined. The concentrations obtained are quite consistent with those provided by conventional radioimmunoassay. The sensitivity can be increased by using a recycling system in conjunction with the TELISA set-up. Alkaline phosphatase is employed as the enzyme label and the immunosorbent is packed in a separate column and positioned in the second enzyme thermistor channel. The substrate, phosphoenolpyruvate, is converted into pyruvate by alkaline phosphatase and the pyruvate is then injected into the recycling column, which raises the sensitivity by a factor of 10–100. Since separate columns are used for detection and the immunosorbent, the sample introduced into the recycling column is very clean, so there is no risk of inhibition or contamination of the column. One other advantage is that several immunosorbent columns can readily be operated simultaneously with one detection column, which allows the cycle time to be shortened by at least five times.

3.3.4 Piezoelectric flow-through immunosensors

The essential requisites to be met by a biosensor are that the electrode surface should remain chemically stable during the measurement process and that a high density of the biological material used be immobilized onto it. However, overloading a piezoelectric crystal with biological material should be avoided at any rate since this may restrict free interaction between the immobilized material and the macromolecular analyte. This is particularly important in piezosensors, where interaction often occurs between antibody and antigen macromolecules. Leakage of immobilized materials during use of the biosensor should also be avoided at all costs, and the immobilized layer should be stable during oscillation. Even though the specialized literature provides a wealth of information on the immobilization and stability of layers deposited on crystal surfaces, the best choice of an immobilization procedure for each particular application cannot yet be predicted.

The use of biological materials as coatings for piezoelectric crystals was first demonstrated by Shons *et al.* [237], who immobilized bovine serum albumin (BSA) on a crystal precoated with a 30% solution of Nyebar C, a low-surface energy plastic. The rationale of using this solution as the coating material is that proteins adsorbed on low-energy surfaces retain their antigenic properties. Exposure of the BSA-coated crystal to a solution

containing anti-BSA resulted in a frequency shift in the coated crystal that was proportional to the concentration of anti-BSA in solution. The sensitivity achieved was equal to or better than that of the passive agglutination method, a conventional procedure used for assaying antibodies. Also, the piezoelectric crystal allowed results to be obtained in a few minutes compared to several hours in the passive agglutination method. The prototypes devised by Shons *et al.* were followed by widespread development of piezobiosensor coatings based on antibodies and antigens. The reaction between the coated crystal and the target analyte is carried out in the liquid phase in all instances, and the ensuing methods are referred to as direct immunoassays.

The early work of Karube *et al.* on piezoimmunosensors was initially aimed at designing batch sensors such as that developed for detection of *Candida albicans* microbes by using anti-*Candida* antibody immobilized onto the surface of anodically oxidized Pd-plated electrodes [238]. Later designs included crystals with a basic resonant frequency of 9 MHz that were modified by immobilizing protein A onto the surface with the aid of (aminopropyl)triethoxysilane. The piezo crystals used for this purpose were positioned inside thermostated flow-cells and the resonant frequency was measured in a continuously flowing stream of de-ionized/distilled water. The resonant frequency shift resulting from the affinity reaction of protein A and human IgG was correlated with concentrations of human IgG in the range 10^{-6} – 10^{-2} mg/mL. The effect of the selective affinity of protein A for each IgG subclass was used to analyse mouse IgG subclass pattern analysis. By means of a stepped gradient of buffer solution, Karube *et al.* identified three different peaks for IgG₁, IgG_{2a} and IgG_{2b}. Rinsing the immunosensor with phosphate–citric buffer allowed each subclass to be gradually separated on the basis of the sensor's resonant frequency change [239].

The determination of dissolved antibodies or antigens by solid-phase radioimmunoassay or enzyme immunoassay involves reaction of a suitable antigen with a solid support onto which it should be immobilized. In this way, picomolar concentrations of antibody can be detected and information on the antibody size and other molecular properties of interest obtained. However, it remains to be seen whether this approach is equally useful for detecting the target analyte in the liquid or gas phase by using an appropriate enzyme or antibody immobilized on a piezo crystal surface. Obviously, a direct comparison between piezoelectric crystal antigen–antibody responses and those of solid-phase radioimmunoassays or enzyme immunoassays is impossible because the latter are essential. Although the binding of anti-

bodies to antigens immobilized on a solid support is well characterized, the nature of the antigen–antibody interaction on the crystal surface remains unknown. This is a crucial matter that requires immediate attention before piezoelectric crystals can be made practical.

3.4 FLOW-THROUGH SENSORS BASED ON AN IMMOBILIZED REAGENT

3.4.1 With reagent consumption

Flow-through sensors based on a non-regenerable immobilized reagent are not the most suitable for use in a continuous regime. The gradual depletion of reagent in the sensing microzone results in a concomitant decrease in the analytical signal in most cases, so adequate reproducibility can only be achieved in a few instances. These sensors normally rely on luminescence techniques and are of the integrated type since placing the sensing microzone on the tip of an optical fibre is inoperative here (the amount of reagent that can be immobilized on the tip of a fibre is so small that the amount consumed in each determination would give rise to a sharp decrease between successive signals).

3.4.1.1 Optical flow-through sensors based on a non-regenerable immobilized reagent

Most of the sensors using a consumable reagent are based on a chemiluminescence phenomenon that is revealed by a reagent such as trichlorophenyl oxalate (TCPO) and a fluorophore (usually a fluoranthene), or luminol.

The research group headed by the late Professor Frei pioneered the development of *flow-through sensors based on immobilized chemiluminescent reagents*. The earliest such sensor used the peroxyoxalate CL system for the determination of hydrogen peroxide [240]. The sensor was essentially non-regenerable since the consumed reagent (TCPO) was not immobilized in the flow-cell, but used in solid form in a previous reactor; the actually immobilized substance was the fluorophore 3-aminofluoranthene, placed in the flow cell depicted in Fig. 3.34.A. The authors assayed several procedures and supports for immobilizing the fluorophore and used the sensor for the determination of hydrogen peroxide in rain water with a detection limit of 1×10^{-8} M. Subsequent variants of the initial sensor lay between those described in this section and Section 3.3.2.1 as they included two reagents

(one regenerable and the other consumable) in the flow-cell. The dual-packed flow-cell shown in Fig. 3.34.B allowed Frei *et al.* to place solid TCPO and the luminophore immobilized on CPG in the detection zone as two consecutive layers. The reproducibility of the resulting system was excellent ($RSD = 3\%$) and the log-log calibration graph run was linear over six orders of magnitude. The sensor's proponents did not specifically state its duration (the repacking frequency must obviously be a function of the analyte concentration in the samples), but pointed out the need for recalibration after repacking owing to the irreproducibility of the packing procedure [241]. The sensor's throughput (100 samples/h) is of little interest for routine analyses taking into account the non-regenerable nature of the sensor.

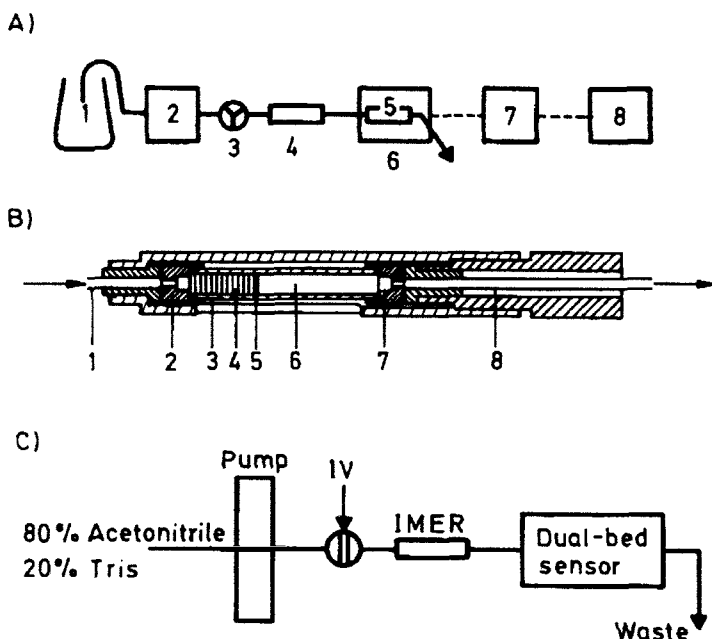


Figure 3.34 — Manifolds for implementation of a sensor containing a packed non-regenerable reagent and a regenerable fluorophore. (A) Flow-through sensor system: 1 eluent vessel; 2 pump; 3 injection valve; 4 TCPO reactor; 5 CL cell; 6 light-tight box with PMT; 7 amplifier; 8 recorder. (B) Design of the packed two-layer sensor: 1 inlet capillary; 2 inlet cap with frit; 3 quartz tube; 4 TCPO layer; 5 frit; 6 luminophore layer; 7 outlet cap with frit; 8 outlet capillary. (C) Manifold for implementation of the previous cell in biochemical applications (Reproduced from [240] and [241] with permission of the American Chemical Society and Elsevier Science Publishers, respectively).

This type of sensor was adapted for biochemical applications by using an enzymatic catalyst (an oxidase) immobilized on CPG and positioned prior to the detector, as shown in Fig. 3.34.C [242].

The above-described two-layer flow-cell was used for the determination of anions based on a quenching phenomenon. Table 3.4 gives the determination limits obtained by using various quenchers and a flow injection manifold in which the cell was inserted. The poor selectivity of quenching can be overcome by using a continuous separation technique (*e.g.* HPLC), as in the determination of the anilines listed in Table 3.4.

Table 3.4. Determinations of anions and amines by luminescence quenching

Species	Technique	LOD (ng)
NO_2^-	FI	2.8
SO_3^{2-}	FI	6.4
thiohydantoin	FI	5.8
thiourea	FI	1.1
ethenyl thiourea	FI	1.6
thioridazin	FI	7.7
sulphoridazine	FI	9.8
methimazole	FI	1.5
aniline	FI	18.0
3-ethylaniline	FI	1.4
4-isopropylaniline	FI	3.5
3,5-dimethylaniline	FI	3.8
N,N-dimethylaniline	FI	3.7
N,N-diethylaniline	FI	3.8
N,N-dipropylaniline	FI	40.0
N,N-dibenzylaniline	FI	> 400
benzylamine	FI	> 400
α -naphthylamine	FI	45.0
2-toluidine	FI	40.0
4-toluidine	FI	47.0
<i>m</i> -methyltoluidine	FI	8.0
4-isopropylaniline	HPLC	5.6
N,N-dimethylaniline	HPLC	2.4
N-ethyl- <i>m</i> -toluidine	HPLC	3.6
N,N-dipropylaniline	HPLC	30.0

Hool and Nieman succeeded in immobilizing the reagent (luminol) on a support (CPG) located in the flow-cell of a chemiluminescence detector. For solubility reasons, the reaction between luminol and glutaraldehyde was

carried out in an ethanol/dimethyl sulphoxide mixture. The loadings thus obtained were 29 μM luminol/g CPG and 86 μM luminol/g silica. There was no evidence of any degradation of the luminol to 3-aminophthalate. The immobilized material was packed in a flow-cell such as that depicted in Fig. 3.35, which was in turn included in a flow injection manifold. The slope of the calibration curve for hydrogen peroxide obtained by using the two types of support was close to unity. The log-log working curves for H_2O_2 were linear over the range 20–600 μM and least-squares analysis provided a correlation coefficient 0.998 and a standard error of estimate of 0.047. Measurements of replicate injections (3–5 per data point) over the entire concentration range provided an RSD of 3% for silica and 4% for CPG. The presence of the bed of particles containing immobilized CL reagent resulted in somewhat degraded precision. Removing the particles and using dissolved luminol instead resulted in an RSD less than 1% and peaks that were 80% broader than those obtained with the particles. HPLC analysis of the flow-cell and a comparison of the chemiluminescence intensities of immobilized luminol and isoluminol showed the former compound to be hydrolysed from the support prior to or during reaction, and emission to occur in solution. One other problem encountered in using glutaraldehyde as the binder for luminol was that arising from particles absorbing a significant amount of chemiluminescence. Over 500 peroxide assays could potentially be done with 1 g of silica containing immobilized luminol [244].

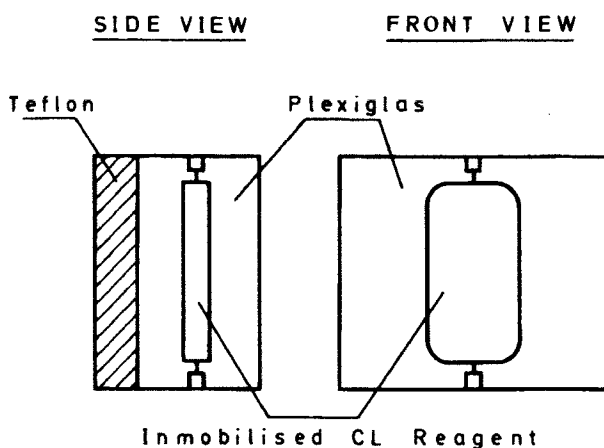


Figure 3.35 — Flow-through chemiluminescence sensor with immobilized luminol. (Reproduced from [244] with permission of the American Chemical Society).

There are other sensors based on non-regenerable luminescent reagents that can be included in flow systems for the determination of oxygen [245], uranyl ion and halides [246]. In the oxygen sensor, the sample diffuses across a hydrophobic O₂-permeable membrane into a reservoir of *tetrakis* (dimethylaminoethylene) (TMAE), with which it reacts to produce chemiluminescence that is measured by a light detection system. The uranyl and halide sensors differ from the oxygen sensor in the fact that the former two require diffusion of the reagent in the sample. In the uranyl sensor, phosphate and nitric acid are diffused into the sample in order to obtain a medium where UO²⁺ ion is fluorescent. In the halide sensor, Ag-fluorescein is diffused into the sample; halides combine with silver ion, thus rendering fluorescein fluorescent.

Absorptiometric sensors can in principle be constructed from sol-gel glasses doped with organic reagents. The sol-gel procedure, an efficient way of producing porous glass at room temperature, can be used to entrap organic reagents into glass matrices without the need for a complicated covalent bonding procedure. Used in an appropriate flow-cell, doped glass can serve as an excellent dispenser for both regenerable and consumable reagents [247].

3.4.1.2 Piezoelectric flow-through sensors based on a non-regenerable immobilized reagent

Most chemical flow-through sensors based on piezoelectric phenomena (measurements of gases or liquids) are of the regenerable type.

A sensor for cyanide based on the formation of the soluble dicyano-gold complex by reaction of the analyte with the metal gold electrode of a piezoelectric crystal was recently reported [248]. The loss of gold was detected by the piezoelectric crystal as a change in the resonant frequency. The sensor was included in a flow injection manifold for automation of analyses. The detection system thus built provides expeditious cyanide determinations with little sample preparation or instrument supervision. However, its detection limits (in the microgram-per-millilitre range) make it unsuitable for real samples. Also, those anions forming soluble and insoluble complexes with gold induce to positive and negative errors, respectively, in the determinations.

3.4.2 With reagent regeneration

While reversible sensors are the ideal sensors, those irreversible sensors containing an immobilized reagent that can be regenerated approach the ideal

performance of a reversible sensor to an extent proportional to the simplicity and expeditiousness with which the reagent can be regenerated, which obviously depends on the particular reagent and analyte, as well as on the interaction between the two that yields the measurable signal. Most of the sensors described in this section are of the regenerable type; some, such as pH sensors based on acid–base indicators, are actually reversible devices. Except for a few sensors based on piezoelectric crystals, all of them rely on optical techniques. Broadly speaking, the mediator-modified electrodes discussed in Section 3.2 can also be included here: they use a reagent (mediator) immobilized on a surface or embedded in a conducting matrix which undergoes a redox transformation by which the electrode process involving the analyte is facilitated, after which the analyte is regenerated. However, because this phenomenon can be considered a particular instance of catalysis, sensors based on regenerable immobilized reagents and electro-analytical detection are included in Section 3.2 rather than this.

Mediated electrochemical sensors aside, there are few sensors involving a reaction at the sensing microzone by which the analyte is not retained to some extent during the time the analytical response is generated. Such is the case with sensors based on luminescence quenching and a few others. Although many of the reactions on which the analytical measurement rests in sensors based on acid–base reactions involve retention of protons, the sensors in question are dealt with in this Section.

3.4.2.1 Optical flow-through sensors based on a regenerable immobilized reagent

Immobilization of a reagent at the sensitive microzone of an optical fibre or the walls of packing material used in flow-cells (indicator-mediated sensors) of non-destructive optical detectors, a major goal in early sensor research, started to be consistently achieved in the 1980s. The earliest attempts involved using integrated sensors [249] and gradually gave way to approaches based on fibre optics.

3.4.2.1.1 Fibre optic-based flow-through sensors

Saari and Seitz [250] used the tip of an optical fibre as the sensing microzone to immobilize a suitable reagent (fluoresceinamine) in order to construct a fluorescence sensor for pH measurements; the design was inspired by previous work of Peterson *et al.*, who used a dye immobilized at the tip of an optical fibre for pH absorptiometric measurements [251]. The

extent to which a sensor of this type is reversible or regenerable depends on the nature of the acid–base indicator used in its construction. The experiments of Saari and Seitz demonstrated the potential feasibility of chemical sensors based on an immobilized fluorogenic reagent. The performance of the pH fluorimetric sensor was found to be liable to improvement in various ways. Thus, the background signal due to scattering was reduced by using a clear film rather than a fine powder as the immobilization substrate, and a system with sufficiently distant excitation and emission wavelengths. The fluorescence intensity of immobilized fluoresceinamine was augmented by using a more powerful excitation source and/or an immobilization system not diminishing the fluorescence efficiency. However, in view of the availability of glass electrodes, the authors aimed at developing sensors responding to metal ions otherwise unmeasurable by potentiometry rather than improving the original pH sensor. More recently, several microsensors based on the same physical and chemical principles were developed after the advantages of optical sensors over electrical based sensors for some applications (particularly in the clinical field) become apparent. Thus, Tan *et al.* reported a near-field optical technique that allows the development of submicro-sized fibre-optical sensors [252]. Their technique involves nano-fabricated optical fibre tips and near-field photoinitiated polymerization. Multi-mode or single-mode optical fibres are drawn into submicro optical fibre tips and then coated with aluminium to form submicro optical fibre light sources. Submicron pH sensors were thus constructed by incorporating fluoresceinamine into an acrylamide–methylenebis(acrylamide) copolymer covalently attached to a silanized fibre tip surface by photoinitiated polymerization. The sensors demonstrated their spatial resolving abilities in measuring the pH of buffer solutions inside micro-sized holes in a polycarbonate membrane. Depending on the sensor size and experimental conditions, the response times ranged from less than 100 to *ca.* 500 ms. In the absence of stirring, the 10–90% response time was *ca.* 300 ms (most probably controlled by diffusion to the sensor).

The frequent need for oxygen measurements of gases and liquids in the industrial, biochemical and clinical fields has fostered the development of a number of sensors based on fibre optics and various chemical principles, most of which rely on the quenching properties of the analyte. The earliest such sensor, reported by Mergman in 1968 [253], used fluoranthene, a strongly fluorescent polycyclic aromatic hydrocarbon (PAH), adsorbed on a porous glass support and excited with a UV light source. The resulting

fluorescence was strongly quenched by oxygen and measured with a photo-cell. Molecular oxygen is known to quench the fluorescence of a variety of other PAHs [254].

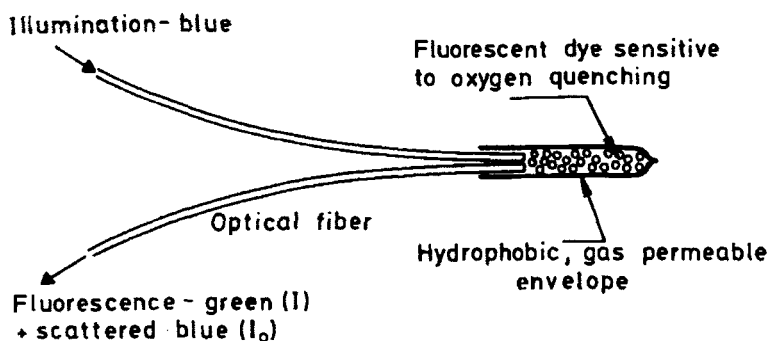


Figure 3.36 — Fibre-optic fluorescence sensor for *in vivo* measurement of oxygen partial pressure. (Reproduced from [255] with permission of the American Chemical Society).

According to Peterson *et al.* [255], a fibre optic flow-through sensor for *in vivo* measurements of the oxygen partial pressure in blood should include three essential elements, namely: (a) a dye with the combined properties of oxygen quench sensitivity, excitation by visible light and resistance to fading; (b) a hydrophobic, highly oxygen-permeable envelope; and (c) an adsorptive, humidity-insensitive support that can activate the dye. On these premises, they built a sensor consisting of two 250- μm strands of plastic optical fibre ending in a section of porous polymer tubing about 3 mm long and 0.6 mm in diameter (Fig. 3.36). The tubing was packed with dye on an adsorptive support. The dye chosen for this purpose was Solvent Green 5, a stable, non-toxic compound available commercially in a high purity, the visible wavelength of which allows the use of plastic optical fibres. Amberlite XAD4 was selected as the most suitable support for the dye on account of its high permeability to oxygen and insensitivity to humidity changes. Also, porous polypropylene was used as the permeable envelope on the grounds of its high gas permeability, mechanical rigidity and strength, and absence of metals potentially posing biocompatibility problems. The end result was a small, inexpensive probe suitable for tissue and blood vessel implantation. While the only other choice of reversible optical indicator, of the absorptiometric type, possibly surpasses luminescence quenching indica-

tors in sensitivity and specificity, no sufficiently stable metal organic compound of this type is seemingly available.

Wolfbeis *et al.* reported a range of biological and chemical sensor designs based on fibre optics and quenching phenomena. The first such sensor, developed for the determination of halides and *pseudo*-halides, was based on the dynamic fluorescence quenching of acridinium and quinolinium indicators, which were immobilized via spacer groups onto a glass surface. The sensors provided the halide concentration in solution via the decrease in the fluorescence intensity arising from quenching. The sensitivity to different halides could to some extent be varied by choosing an appropriate indicator. Sensor responsiveness increased from chloride to iodide and the detection limits achieved were 0.15, 0.40 and 10.0 mM for iodide, bromide and chloride ion, respectively. The reversibility of the sensor response was investigated by pumping halide solutions through the sensing unit until the signal was constant and then switching to water or 0.1 M sodium sulphate. The average response time for a signal change to be indicated by 95% of its signal was *ca.* 40 s [256].

This group extended their research into chemical sensors to biosensor development by devising a more complex modification of optical fibres in order to have them take part not only in the typical detection reactions dealt with in this Chapter, but also in the series of steps illustrated in Fig. 3.3.A, namely: (a) a glucose oxidase (GOD)-catalysed reaction at the surface of the catalyst supporting membrane; (b) biochemical depletion of the dissolved oxygen diffusing across the membrane; and (c) quenching of the fluorophore fluorescence by the oxygen [257]. Because this type of sensor involves both separation and a dual reaction, it is also commented on in Chapter 5.

A similar sensor using GOD immobilized on carbon black gel (Fig. 3.4.B) was developed by Schmid's group [258] for routine determinations of glucose in wines and fruit juices. The sheet of carbon black used instead of a nylon membrane was additionally intended to serve as the enzyme support (specifically, as optical insulation to protect the oxygen optrode from ambient light and the sample fluorescence). The indicator used, decacyclene, was dissolved in a 25- μm thick film of silicone rubber and attached to a 100- μm thick polyester support. The biosensor was inserted in a flow injection manifold for measuring glucose at concentrations between 0.1 and 500 mM with an RSD of 2.0% at 60 samples/h. It proved to remain stable after over 400 h of continuous use. A similar biosensor using lactate oxidase

instead of GOD was employed by the same authors for the on-line determination of lactic acid during kefir fermentation [259].

The research activity of Wolfbeis *et al.* into the interference of oxygen with the determination of halothane and their search of a specific sensor for this anaesthetic led to the construction of a fibre-optic flow-through sensor based on the quenching effect of oxygen on the anaesthetic's fluorescence that allowed both to be determined. Various indicators and polymers were tested for construction of sensitive membranes in order to select that providing the greatest analyte discrimination. The three basic types of combinations assayed involved (a) two sensor layers containing the same indicator (decacyclene) in two different polymeric solvents (silicone rubber and polyisoprene/dioctyl phthalate); (b) two layers of the same composition [one coated with a 12- μm thick film of polytetrafluoroethylene (PTFE), which is impermeable to halothane]; and (c) two sensor layers loaded with the same indicator (decacyclene) in the same polymeric solvent (silicone rubber), one of which was coated with PTFE to prevent quenching of halothane. The results provided by combinations *b* and *c* above showed halothane and oxygen determinations to be feasible over a wide concentration range with adequate precision. Combination *c* was found to excel *b* for two reasons, viz. (i) both sensor layers were prepared in exactly the same way—except that one was coated with PTFE—, which is quite desirable when manufacturing layers on a large scale; and (ii) only two quenching constants need be determined in the calibration procedure. In fact, combination *c* allowed the determination of halothane, oxygen or both, with a precision of 5% for the anaesthetic and 3.5% for oxygen in the typical concentration ranges for both. The probe proved to be virtually specific to the two analytes since other gases present in inhalation gases or blood (carbon monoxide, dinitrogen monoxide and fluorans included) posed no interference [260].

Barnard and Hawkins developed one other fluorescence flow-through sensor for halothane. Their probe uses a poly(ethyleneglycol) film containing two fluorophores, viz. 2,5-diphenyloxazole and *tris*[4,4,4-trifluoro-1-(2-thienyl)butane-1,3-dione]europium(III). The film fluoresces strongly with a red line spectrum on excitation in the range of *ca.* 200–380 nm. Evidence for direct energy transfer between the two fluorophores was provided. While the sensor response does not obey the Beer–Lambert law, the sensor can be used to determine the anaesthetic over the medically significant concentration range, viz. 0–3%. No study of the potential interference of other quenchers with the sensor response was reported [261].

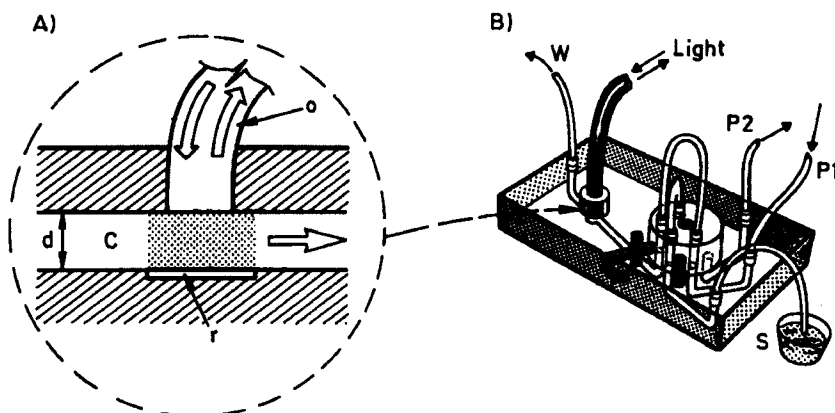


Figure 3.37 — (A) Optosensor with fibrous flow-through structure on the surface of which a pH indicator is covalently bound: C Carrier stream; d thickness of indicator-containing cellulose pad; r reflector; o optical fibre. (B) Integrated microconduit for measurement of pH comprising injection valve and optosensor: S sample solution, P1 and P2 tubes leading to the peristaltic pumps; W waste tube. (Reproduced from [262] with permission of Elsevier Science Publishers).

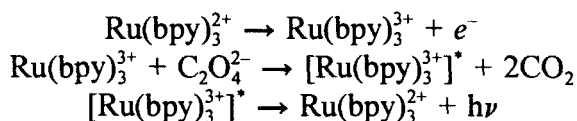
Ruzicka and Hansen used reflectance spectrophotometry as the basis for pH flow injection measurements in order to demonstrate the principle behind and test the performance of microconduit-integrated optosensors [262]. The proposed flow-through optosensors (Fig. 3.37.A) used a commercially available fibrous cellulose material to which acid–base indicators were covalently bound and through which the carrier stream (C) was allowed to pass freely so that the change in the reflectance of the incident light, led to the fibrous pad by a bifurcated optical fibre (o), might be measured at the distant end by means of an ordinary spectrophotometer. If the pad thickness (d) were to be sufficiently great, then no reflector (r) would have been needed because each cellulose fibre, being white, served as a diffuse reflector. However, for miniaturizability (the channel thickness was 2 mm), an "infinitely thick layer" was unfeasible. Several acid–base indicators covalently bound to the surface of cellulose fibres were tested with the aim of optimizing the reproducibility of a series of pH measurements, maximum range and maximum sensitivity of pH measurements, the reproducibility of the optosensor preparation procedure and the long-term stability of the optosensor calibration curves, as well as the potential influence of colour, the refractive index and the presence of air bubbles in the measured sample solution. The

resulting microconduit flow system (Fig. 3.37.B) consisted of $70 \times 45 \times 10$ mm PVC blocks into which appropriate channel patterns were impressed or engraved. When closed by a transparent cover with the aid of pressure-sensitive polymeric glue, the channels formed conduits of semi-circular cross-section with an internal area of 0.8 mm^2 [263]. These microconduit-integrated optosensors proved to be useful for the routine sequential determination of various acids and bases [264], as well as pH measurements of rain water [265]; the throughput for an injected sample volume of $30 \text{ }\mu\text{L}$ was 120 samples/h.

3.4.2.1.2 Integrated flow-trough sensors

All integrated sensors based on an interaction between the analyte and reagent (neither of which is used in a retained form) and regeneration of the latter rely on chemiluminescent reactions involving electroregeneration of the reagent or a quenching phenomenon. On the other hand, absorptiometric and reflectometric sensors of this type use colorimetric acid–base indicators supported on a suitable material.

Nieman *et al.* developed an unusual sensor for readily oxidized compounds. The underlying chemical principle was previously exploited in a continuous-flow configuration for the determination of alkylamines in which the reagent, dissolved *tris*(2,2'-bipyridine)ruthenium(III), was merged with a buffered stream into which the sample was injected. The redox reaction between the amine and the complex yielded the reduced form of the latter in its excited state, which was strongly luminescent [266]. Downey and Nieman used this reaction as the basis for a sensor in which the reagent was regenerated electrochemically by immobilizing the reduced form of the complex $\text{Ru}(\text{bpy})_3^{2+}$ on a Nafion film deposited on an electrode as shown in Fig. 3.38.A. The reaction scheme for the electrogenerated chemiluminescence (ECL) from the ruthenium complex and oxalate



demonstrates that the starting reagent was regenerated. The sensor was used in a flow injection manifold to determine oxalate, alkylamines and NADH with detection limits of $1 \text{ }\mu\text{M}$, 10 nM and $1 \text{ }\mu\text{M}$, respectively, and working concentration ranges spanning 4 decades. The sensitivity was constant over a wide pH range (from 3 to 10). With oxalate and, to a small extent, amines,

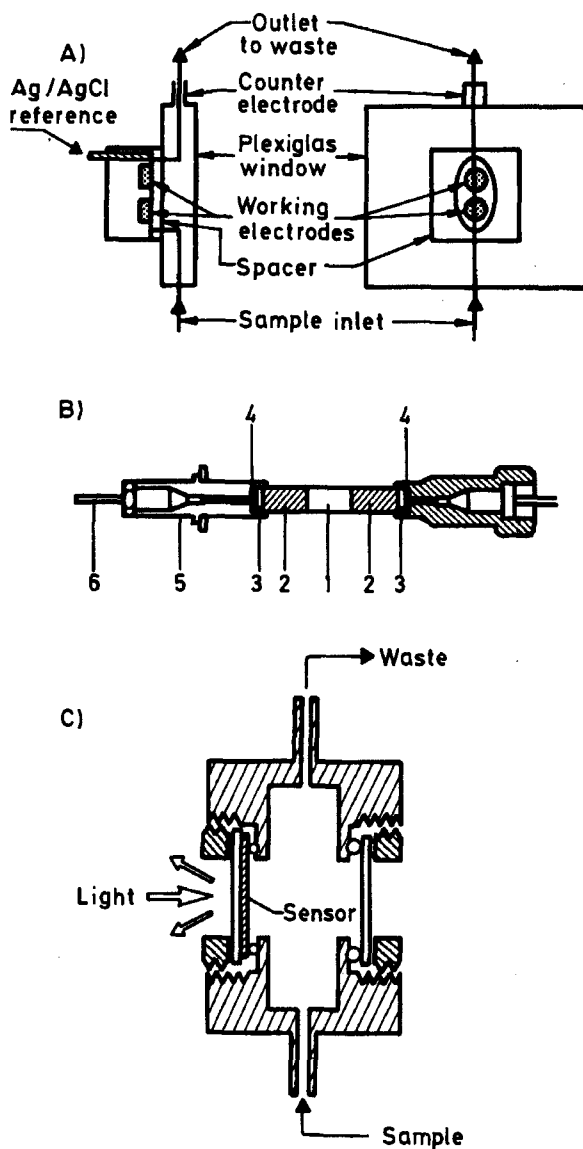


Figure 3.38 — Integrated flow-through sensors. (A) With electrochemical generation of the luminescent reagent. The flow stream path follows the line between the analyte inlet and the outlet to waste. (B) With immobilization of a phosphor (length, 3 cm; internal diameter, 2 mm): 1 immobilized phosphor; 2 CPG; 3 quartz wool plug; 4 KEL-F caps; 5 hand-tightened screw; 6 stainless steel capillaries. (C) Sensor based on reflectance measurements. The sensor membrane is fixed on a Plexiglas disc. Reflectance spectra are measured from the rear side. (Reproduced from [267] and [269] with permission of the American Chemical Society and Elsevier Science Publishers, respectively).

emission intensities were found to increase with increasing ionic strength; this was shown to be related to the Nafion film rather than the chemiluminescent reaction. The sensor remained stable for several days if stored under appropriate conditions [267].

In addition to the sensors dealt with in Section 3.3.1.1, which could equally have been included in this Section as they use consumable immobilized reagents and regenerable fluorophores, Frei *et al.* developed a sensor for HPLC determinations based on the solid-state detection cell depicted in Fig. 3.38.B, where they immobilized 1-bromonaphthalene for measuring phosphorescence quenchers. Experiments demonstrated the sensor's usefulness for determining nitrate with a detection limit of *ca.* 10^{-7} M and an RSD of 4% for an analyte concentration of 10^{-6} M. However, the scope of application of this sensor to chromatographically separated anions is rather narrow owing to the low sensitivity of the quenched phosphorescence detection for iodide and other halides [268].

Reflectance measurements provided an excellent means for building an ammonium ion sensor involving immobilization of a colorimetric acid–base indicator in the flow-cell depicted schematically in Fig. 3.38.C. The cell was furnished with a microporous PTFE membrane supported on the inner surface of the light window. The detection limit achieved was found to depend on the constant of the immobilized acid–base indicator used; it was 10^{-6} M for *p*-Xylenol Blue ($pK_a = 2.0$). The response time was related to the ammonium ion concentration and ranged from 1 to 60 min. The sensor remained stable for over 6 months and was used to determine the analyte in real samples consisting of purified waste water, which was taken from a tank where the water was collected for release into the municipal waste water treatment plant. Since no significant interference from acid compounds such as carbon dioxide or acetic acid was encountered, the sensor proved to be applicable to real samples after pH adjustment. The ammonium concentrations provided by the sensor were consistent with those obtained by ion chromatography, a spectrophotometric assay and an ammonia-selective electrode [269].

3.5 FLOW-THROUGH SENSORS BASED ON AN *IN SITU* PRODUCED REAGENT

Some continuous systems integrate reaction and detection in a flow-cell, but no catalyst or reagent is immobilized —rather, the reagent is produced in

the cell itself. This type of sensor system thus shares some characteristics of those dealt with in this Chapter, yet its peculiarities call for separate discussion. In any case, they should not be confused with those described in the previous section, where the immobilized reagent is regenerated but not produced *in situ* in any case.

No mention is obviously made here of those dynamic systems where reaction and detection are integrated by virtue of the fact that the sample and reagent are mixed at the detection point, which is usually imposed by the chemical system itself (*e.g.* in bio- and chemiluminescent reactions, where deactivation after reaction is so rapid that the latter has to be simultaneous with detection [270]) or by the type of measurement used (*e.g.* kinetic stopped-flow measurements, which require monitoring of the early stages of reaction [271]). This Section is therefore exclusively devoted to those sensors in which reaction is integrated with detection because it is at the sensing zone that the reagent "appears" in a suitable form for the analyte to be derivatized. The external energy that facilitates *in situ* development of the reaction can be used to regenerate the reagent (Section 3.5.1) or as a reaction ingredient (Section 3.5.2).

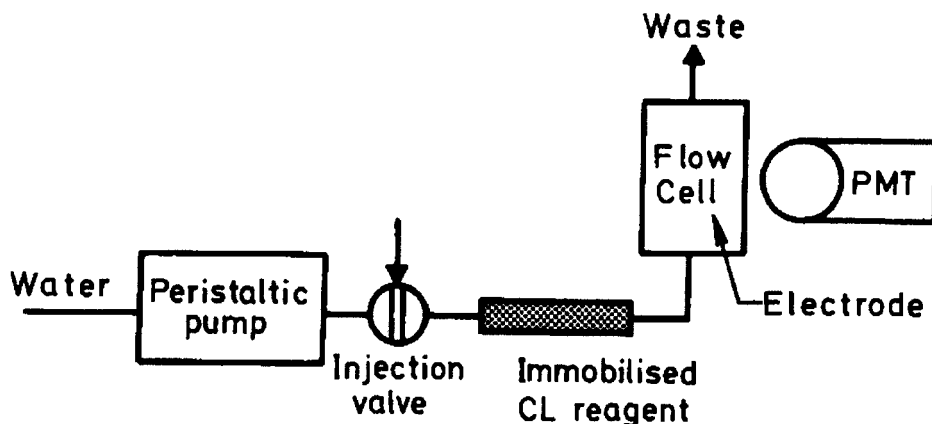


Figure 3.39 — The sample (hydrogen peroxide/buffer) is injected into a flowing stream of water and flows through a reservoir where a small amount of luminol is cleaved from the support. The peroxide/buffer/luminol plug then enters into the cell containing the electrode. The luminol reaction proceeds and the luminescence is measured by a PMT. (Reproduced from [76] with permission of the American Chemical Society).

3.5.1 Reagent generation with the aid of external energy

The active form of a reagent is most often generated via a redox process. The different types of devices applied to a flow-cell in order to generate the reagent required have led to as many types of sensors.

The luminol reaction has long been known for its use in H_2O_2 determinations in flowing systems: the reagent is oxidized in an alkaline solution in the presence of a catalyst to form 3-aminophthalate in an excited state. The reaction can be made *pseudo* first-order in the H_2O_2 concentration so that the emitted light intensity is directly proportional to the analyte (peroxide) concentration. The catalyst used can be a biological substance (*e.g.* the peroxidase used in the solid-state sensors developed by Nieman's group [75,76]), but also an electrode effecting the one-electron oxidation of luminol and subsequent further oxidation of the intermediate produced by a solution phase oxidant such as oxygen or hydrogen peroxide. Because oxygen is normally present in aqueous solutions, the hydrogen peroxide (analyte) generated CL is measured above a background. The oxidation of luminol at a gold electrode takes place at *ca.* +0.25 V vs Ag/AgCl. The emission is confined near the electrode surface. Nieman *et al.* proposed a detection scheme for peroxides based on the luminol reaction and entirely immobilized or "solid-state" reagents. In such a scheme, all reagents are immobilized and no reagent solution is required (see Fig. 3.39). Hydrogen peroxide is injected into a stream of 0.2 M KNO_3 (the supporting electrolyte) together with buffer, and flows into a reservoir where the electrode is immersed. The sensor performance was compared with that of another previously developed by Nieman's group for the same analyte using peroxidase immobilized in a flow-cell and found the electrode to provide the better detection limit (*ca.* 100 pmol) and working range [75,76].

One way of indirectly generating the reagent is by obtaining a species that can convert it into an active form. Such is the case with sensor developed by Wang and Yeung, where hydroxyl ion, a reactant in the luminol chemiluminescence system, is produced by reduction of hydrogen peroxide in the direct-current electrolytic flow-cell depicted in Fig. 3.40. Luminol CL is therefore generated in an indirect manner. The sensor can be used in both HPLC and FI systems as follows: the mobile phase (or carrier) is used to carry the luminol, hydrogen peroxide, sodium chloride and Co(II). Some H_2O_2 is reduced to OH^- ion in the electrolytic cell. As the eluent flows through the cell, the OH^- produced from the cathode establishes an alkaline environment that is suitable for H_2O_2 to oxidize luminol and produce the

chemiluminescence. On being eluted, the analyte displaces other components of the eluent and decreases the CL intensity, giving rise to a negative peak. Injection of the analyte into an FI manifold including the sensor has a similar effect. Consequently, the system allows the determination of species with no CL properties, usually considered to be undetectable chemiluminometrically [272].

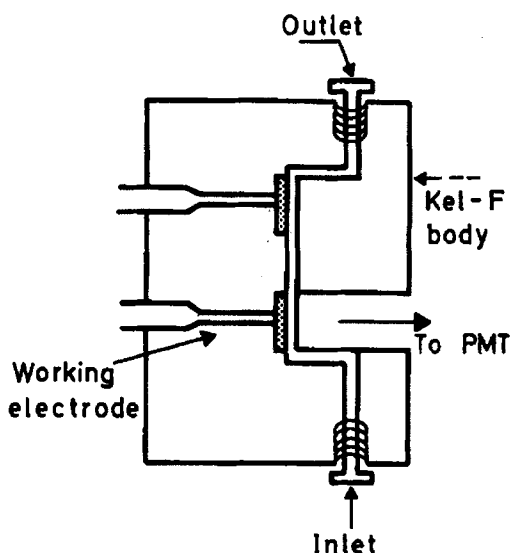


Figure 3.40 — Sensor for the indirect chemiluminescence determination of species based on electrode generation of OH^- ions. (Reproduced from [272] with permission of Elsevier Science Publishers).

Several coulometric sensors involving generation of a reactant in a flow-cell by application of an exact amount of electricity have been reported. The group headed by Ruzicka and Christian reported an FI system where a gradient chamber, a reagent regeneration chamber and a fibre optic-assisted photometric flow-cell were integrated as shown in Fig. 3.41.A for implementation of stopped-flow coulometric titrations [273]. The gradient chamber was a piece of 10-mm inner diameter glass tubing with PTFE end caps that was furnished with a miniature stirring bar (see detail in Fig. 3.41.A). The net chamber volume could be varied by adjusting the end cap positions. The generating and counter-electrodes were 1.2-mm diameter platinum wires. The 3.4-cm long generating electrode was coiled around the bottom of the mixing

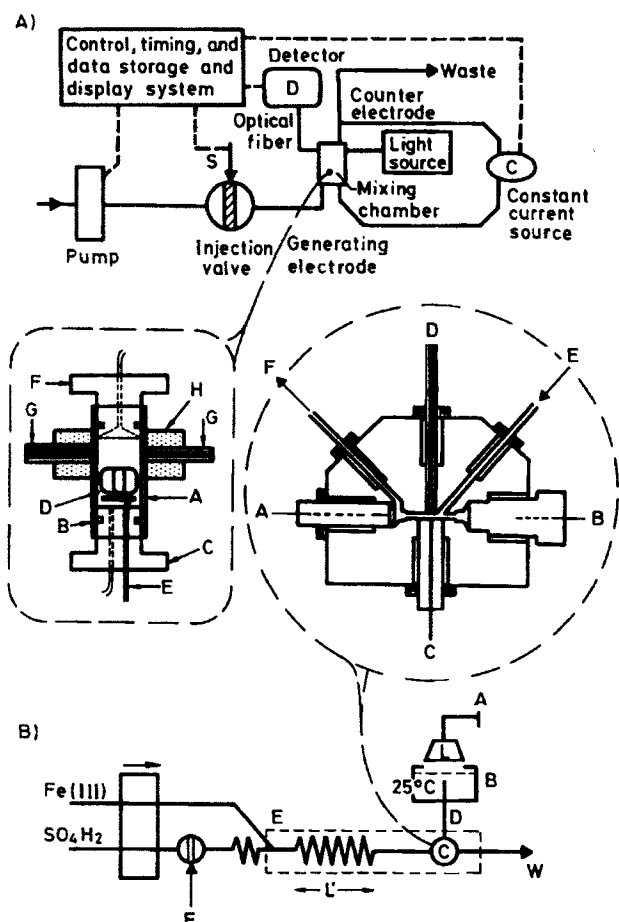
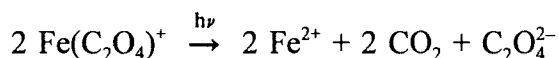


Figure 3.41 — (A) Stop-flow coulometric titration system. Light is transmitted from the source, across the gradient chamber, onto the detector via optical fibres. The injection valve pump and constant current source are computer-controlled. Detail of the integrated flow-cell, gradient chamber, and reagent generation chamber. The chamber body (A) is 10-mm ID glass tubing. The PTFE end caps are sealed with O rings (B). The chamber is mounted vertically with the inlet cap (C) on the bottom. The star-shaped stirring bar (D) rests atop the coiled generating electrode (E). Light is transmitted across the chamber and collected by optical fibre bundles (G). (Reproduced from [273] with permission of Pergamon Press). (B) Manifold for implementation of a photochemical sensor: L radiation source; A intensity regulator; B thermostated water bath; C electrochemical cell; D optical fibre bundle; E light-tight box; F injection valve; L' reactor; W waste. Detail of the laboratory-made electrochemical flow-cell: A, B and C auxiliary, reference and working electrode, respectively; D optical fibre; E and F flow inlet and outlet. (Reproduced from [274] with permission of Elsevier Science Publishers).

chamber, with an overall surface area of 1.3 cm^2 . The counter-electrode was placed in a separate chamber downstream of the mixing chamber. The connecting tubing was 4-cm long. Light was directed from an external source via a 45-cm long \times 5.56-mm diameter optical fibre bundle, across the chamber, and collected and directed to a spectrophotometer via another, 45-cm long \times 3.18-mm diameter optical fibre bundle. The titration procedure involved the following steps: (a) a sample or blank (H_2O) was injected into the carrier containing the electrolyte and indicator; (b) at a preset delay time, the flow was stopped, causing a selected portion of the sample to be arrested in the chamber; (c) at that point, a current was applied that produced a reagent for the titration; (d) a titration end-point was reached and the current stopped; (e) after long enough for the end-point to be reached, the flow was resumed to flush the chamber out. This automated sequence of events was controlled by a computer furnished with software for display of the first derivative of the flow-injection recording, which was used to determine the inflection point of the titration curve and hence Δt . The end-point was detected at $\lambda = 620 \text{ nm}$. By changing the polarity of the constant-current source, hydroxyl ions or protons were produced in the mixing chamber, depending on the nature of the analyte. The direct proportionality of the measured parameter (Δt) to the concentration of the sample portion assayed, rather than the logarithm of the sample concentration (typical of FI titrations), resulted in increased precision. Because the titrant was produced within the system during the determination, the typical problems associated with reagent handling, storage and stability were avoided. Since unstable reagents can also be produced by electrolysis, the sensor can be used to exploit a wide range of chemistries [273].

3.5.2 Use of light as a reaction ingredient

In contrast with the sensors described elsewhere in this Chapter, the device proposed by the authors' group uses no reagent, but photons, to induce a photochemical reaction, and involves electrochemical detection of the photochemical product, which allows one to continuously monitor the formation of the electroactive product. Kinetic monitoring increases the selectivity of determinations by eliminating matrix effects and the contribution of side reactions, whether slower or faster than the main reaction. The electrochemical system chosen for implementation of this special sensor was the $\text{Fe(II)}/\text{C}_2\text{O}_4^{2-}$ couple, which was used for the kinetic determination of oxalate ion based on the following reaction:



The course of the reaction was monitored amperometrically at +0.900 V via the oxidation of Fe(II). Figure 3.41.B depicts the flow injection manifold and flow-cell used. The sample was injected into a 0.1 M H₂SO₄ carrier stream that was merged with the solution of Fe(III), with which it mixed along reactor L, the mixture being subsequently propelled to the electrochemical cell. Both the reactor and the cell were housed in a metal box to protect them from light other than from the lamp. A strand of optical fibres (D) was used to guide the light from the lamp to the flow-cell. The ends of the fibres were plunged in a thermostated bath, the water level of which was kept 0.5 cm above the fibres, which were separated 16 cm from the centre of the lamp. The lamp was switched on and at maximum intensity during the measurement time only in order to avoid local overheating. Each sample was injected in triplicate and non-irradiated blanks were also measured under the same working conditions. Application of the initial-rate kinetic method provided excellent results, as can be seen from Table 3.5. Other kinetic alternatives such as the fixed-time method provided larger errors [274].

Table 3.5. Analysis of synthetic samples of oxalate by the initial-rate method

Added (µg/mL)	Found (µg/mL)	Error (%)
4.69	4.79	2.1
6.70	6.63	-1.0
9.38	9.43	0.5
10.70	10.66	-0.4
14.70	14.35	0.3
20.10	20.14	0.2
40.20	40.16	-0.1

Sensors based on integrated reaction and detection are the most varied and numerous among flow-through sensors and those that will predictably experience the greatest development in the near future. Both enzyme sensors and immunosensors are bound to become virtually irreplaceable tools in some areas of social interest including clinical and environmental analysis. While other sensors inspired by those discussed in Sections 3.4 and 3.5 may

allow specific problems to be solved, they have much less ground for development and application.

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4

Flow-through sensors based on integrated separation and detection

4.1 INTRODUCTION

Non-chromatographic continuous separation techniques [1–6] are appealing alternatives to their batch counterparts, which they surpass in compatibility with continuous analysers, precision, sample and reagent economy, throughput, scope of application and affordability. They also offer some additional advantages such as the ability of indirectly enhancing sensitivity and selectivity by preconcentration and sample clean-up, respectively. The variety of interfaces available between separation techniques, which include gas–solid, gas–liquid (gas diffusion), liquid–liquid (dialysis, extraction) and liquid–solid (sorption, electrochemical stripping) interfaces, have fostered the development of sensitive and selective enough methods for application to real samples. The separation process can involve the analyte, its reaction product (separation–preconcentration) or some potential interferent (clean-up). In any case, using an appropriate separation unit prior to detection allows the analyte to reach the detector under optimal conditions for concentration and isolation.

One of the most topical ways of approaching this type of system, where separation and detection take place sequentially in space and time, to current trends in Science and Technology (*e.g.* automation and miniaturization) involves integrating both steps. Integrated systems of this type meet the requirements of chemical sensors [7,8] and differ clearly from conventional flow systems, where detection and mass transfer take place at different locations in the continuous configuration. In fact, the characteristic mass transfer of separation techniques takes place simultaneously with detection

in this type of sensor. Unlike sensors based on integrated reaction and detection (Chapter 3), where the sensor sensitivity and responsiveness are determined by the kinetics of the reaction involved, the properties of the sensors dealt with in this Chapter are governed by the kinetics of mass transfer. Also, unlike the sensors discussed in the previous and following chapter, these never involve a chemical reaction.

Integrated separation–detection systems conforming to the definition of sensor established in Chapter 2 may involve three types of interface, *viz.* gas–liquid (gas diffusion), liquid–liquid (dialysis, extraction) and liquid–solid (any of the forms in which the analyte or a reaction product may react with a solid support, *i.e.* adsorption, ion exchange, *etc.*). Other separation techniques involving one of these types of interface do not allow implementation of systems strictly fitting the definition of sensor. Thus, concentration and detection in stripping techniques coincide in space but clearly not in time, so they can never be used as the basis for implementation of sensors proper. There are also some liquid–liquid extraction systems without phase separation where the extracting phase is monitored in a continuous fashion, which is equivalent to following the kinetics of mass transfer between the two liquids [9–11]. These systems are too complex for complying with the definition of sensor. Finally, systems involving simultaneous gas diffusion and atomic spectroscopic detection (*e.g.* mercury vapour formation or hydride generation and subsequent diffusion across a membrane located in the detection zone [12–15]) also fail to meet sensor requirements as they cannot be miniaturized readily.

The above exceptions leave relatively few sensors based on integrated separation and detection, particularly of the types involving gas–liquid and liquid–liquid interfaces, which require the detector to be responsive to the gas or ion (molecule) transferred across the membrane. The scope of liquid–solid interactions is somewhat broader as it enables not only retention of the analyte and monitoring of some intrinsic property, but also to retain a product of a previous reaction, thereby substantially expanding the possibilities.

The sensors discussed in this Chapter are dealt with in five sections. The first three (Sections 4.2–4.4) are devoted to single-parameter sensors, which are dealt with in increasing order of use (*viz.* those involving a gas–liquid, liquid–liquid or liquid–solid interface). A further distinction is made according to the type of detector involved. Section 4.5 is concerned with multi-parameter sensors, which are still unfortunately much less common,

even though there is room for future improvement via two different routes: (a) use of discriminating sensors or (b) inclusion of a separation step prior to detection. Finally, Section 4.6 deals with ion-selective electrodes (ISEs) and those based on field-effect transistors (ISFETs). While these sensors do not integrate separation proper with detection, they somehow involve mass transfer across a membrane (ISEs) or retention (ISFETs), so they warrant inclusion in this Chapter. Leaving these types of sensor out of a book on flow-through sensors would have been unjustifiable, however difficult they may be to classify. Figure 4.1 shows several possible classifications for the sensors dealt with in this Chapter based on various criteria.

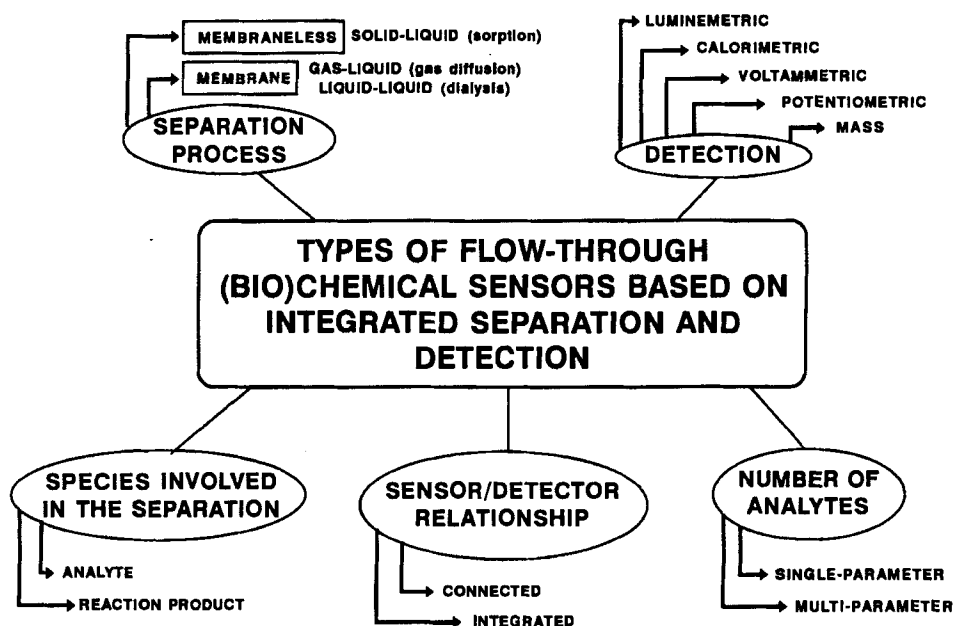


Figure 4.1 — Classification of flow-through (bio)chemical sensors integrating separation and detection according to various criteria.

4.2 INTEGRATED GAS DIFFUSION AND DETECTION

Exclusion of gas-diffusion systems integrated with atomic detectors [12–15] for the above-mentioned reasons leaves very few sensors that rely on integrated diffusion and detection as gaseous analytes lending themselves

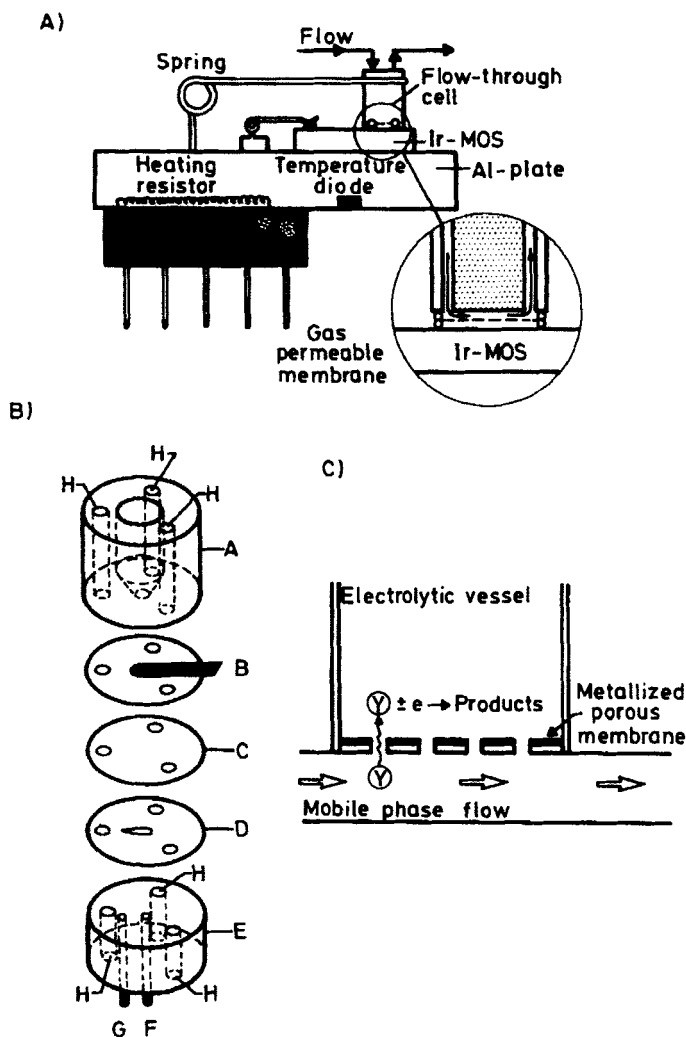


Figure 4.2 — (A) Schematic diagram of an ammonia-N-sensitive probe based on an Ir-MOS capacitor. (Reproduced from [20] with permission of the American Chemical Society). (B) Pneumato-amperometric flow-through cell: (a) upper Plexiglas part; (b) metallized Gore-Tec membrane; (c) auxiliary Gore-Tec membrane; (d) polyethylene spacer; (e) bottom Plexiglas part; (f) carrier stream inlet; (g) carrier stream outlet. (C) Schematic representation of the pneumato-amperometric process. The volatile species Y in the carrier stream diffuses through the membrane pores to the porous electrode surface in the electrochemical cell and is oxidized or reduced. (Reproduced from [21] with permission of the American Chemical Society).

readily to direct detection are very scarce and gas-selective electrodes are dealt with in Section 4.6. A description of two such sensors follows.

One of them is based on semiconductor technology, which allows construction of small, inexpensive, reliable sensors that can be directly integrated with control and data-handling electronic circuits. The sensor uses an ammonia-sensitive metal oxide semiconductor (MOS) design [16,17] and a thin plate of catalytic metal such as iridium as a part of the gate. Iridium-metal oxide semiconductor (Ir-MOS) capacitors are highly sensitive to gaseous ammonia (the detection limit in air is *ca.* 1 ppm/vol or 0.59 mg/kg) and can be manufactured with rather good reproducibility. Figure 4.2.A depicts a probe of this type. The IR-MOS capacitor ($5 \times 10 \text{ mm}^2$) is placed on a $2 \times 7 \times 20 \text{ mm}^3$ aluminium plate with a ground niche intended to ensure correct sensor positioning. The aluminium plate is thermostated at 35°C by means of a temperature-sensing diode and a heating resistor placed on the backside of the aluminium plate. A circular flow-through cell (5 mm diameter, 0.2 mm deep) is positioned at the edge. The cavity is connected to an inlet and outlet that are placed diametrically to let buffer pass through. A gas-permeable membrane (PTFE, pore size $1 \mu\text{m}$, diameter 5 mm) and a Teflon gasket (3 mm ID, 5 mm OD, 0.1 mm thickness) are mounted over the cavity. The flow-through cell is pressed against the active surface of the sensor by a spring to ensure tight sealing, mounted on a 5-pin integrated circuit socket and electrically connected to a C-V meter and a temperature control circuit. Ammonia molecules in equilibrium with ammonium ions in solution diffuse across the gas-permeable membrane and are detected by the sensor. This phase-separation stage has the advantage that the sensor is thereby electrically insulated from the solution. Background noise is thus decreased, so use of a reference electrode is unnecessary. This ammonia-N probe (ammonia-N denotes the sum of ammonia molecules and ammonium ions in solution) has proved to be useful for the determination of the analyte in various biological and non-biological solutions such as whole blood, blood serum, rainwater and river water [18]. The probe was used in combination with an immobilized urease reactor to develop a method for the determination of urea [19] and a flow-injection method for quantitation of creatinine in biological fluids using creatinine iminohydrolase (CIH) immobilized onto CPG in a reactor located prior to the detection system [20]. The amount of ammonia released in the enzyme-catalysed reaction was a measure of the creatinine concentration. The normal background level of ammonia-N in biological samples such as whole blood or urine is roughly similar to or

higher than that of creatinine. Consequently, endogenous ammonia-N in a sample has to be removed prior to the determination of creatinine, which can be accomplished by co-immobilizing glutamate dehydrogenase with CIH. In this way, endogenous ammonia-N is simultaneously removed and the need for blank correction is avoided.

Trojanek and Bruckenstein developed a flow-through pneumato-amperometric sensor for the determination of nitrite based on a gas-permeable Gore-Tex membrane, one face of which was covered with a porous gold electrode [21]. The flow-cell (Fig. 4.2.B) consisted of two Plexiglas parts held together by three screws. The upper part served as the electrochemical cell and contained 0.1 M sulphuric acid, a gold wire auxiliary electrode, and a capillary leading to a saturated calomel reference electrode. Electrolyte contact to the porous gold working electrode was made through a 0.1-in. diameter hole drilled in the bottom of the upper part. The metallized gold membrane is sealed to the cell bottom around the hole using two layers of pressure-sensitive silicone adhesive through which a 0.1-in. hole was punched. This assembly provides a well-defined working electrode area in contact with the sulphuric acid electrolyte without any leakage of electrolyte from the cell. The bottom part of the detector is screwed tightly to the upper half of the detector, thereby clamping together the metallized membrane, the auxiliary membrane, and the 0.015 in. thick polyethylene spacer cut out to connect the liquid inlet and outlet, both of which were attached to the bottom half of the cell. The most severe shortcoming of earlier pneumato-amperometric sensors [22,23] was the long time required for the monitored species to pass from the liquid to the gas phase, which resulted in sluggish determinations. This shortcoming was circumvented by using the set-up depicted in Fig. 4.2.C, where X was reacted with R to form Y, and the solution containing X, R and Y is circulated over the gas porous electrode surface. The unmetallized Teflon face of the membrane contacted the aqueous liquid phase containing R, any unreacted X, and Y. This last, which was a volatile electroactive species, partitioned into and through the gas pores in the membrane and reached its metallized face. The potential of the porous metal electrode was selected to be in the limiting current region for electrolysis of Y. Hence, the electrolysis current was proportional to the amount of X from which Y was produced. It should be noted that, even if R was electroactive, it yielded no current provided it was non-volatile. The response time of this sensor is sufficiently fast to permit flow-injection determinations at a rate as high as 100 samples/h.

4.3 INTEGRATED LIQUID-LIQUID SEPARATION AND DETECTION

Flow-through sensors based on integrated optical detection and a liquid-liquid separation are relatively scant since the analytes are rarely determined by their photometric or luminescence properties. Thus, with few exceptions, these sensors use amperometric detection —as noted earlier, ISEs and ISFETs are dealt with separately in Section 4.6.

These sensors use two chief types of separation techniques involving a liquid-liquid interface, *viz.* dialysis and liquid-liquid extraction —the latter, despite its potential for future expansion, has only been used with a single sensor, though.

4.3.1 Sensors based on integrated dialysis and detection

Dialysis, which involves mass transfer between two miscible liquids separated by a membrane, has been widely used in continuous systems [1], particularly in clinical analysis as a means for separating macromolecules present in biological fluids from the species of interest (of generally low molecular weights).

Olson's group reported the first attempt at integrating dialysis with optical detection. They used a pulsed laser as the light source and a specially designed cell for studying mass transfer across membranes with no need for sample extraction, since spatially resolved data are acquired from the solution on both sides of the membrane. The system provided an instantaneous measurement or "snapshot" of the amount of substances that had passed from one side of the membrane onto the other. For example, concentration-time profiles for ferricyanide ion during electrolysis of the solution above a graphite electrode were acquired as a function of time. The system was automated for measuring concentration-distance profiles at programmable times. From the spectroelectrochemical data obtained it was apparent that the laser-photodiode array sensor could measure concentrations as a function of both time and distance. The results obtained were consistent with the theoretical predictions. Even though the system was only applied to quiet solutions, a minor alteration of the measuring cell should enable flow measurements and hence dynamic monitoring. In this way, information could be acquired by monitoring both the analyte and those species in the sample that might disturb its transfer across the membrane [24].

A number of membrane-coated voltammetric electrodes are based on integrated *dialysis* and detection. The inherent selectivity achieved by having the analyte pass through a membrane of a given cut-off, which allows the

species of interest to be isolated from at least those of sizes exceeding the membrane pore size, is furthered by the use of permselective membranes as electrode coats on account of their special properties.

Wang *et al.* have used this type of membrane to design a number of amperometric flow-through sensors. Their earliest designs involved a glassy carbon electrode (GCE) covered with a cellulose acetate film, access to which surface was regulated via controlled basic hydrolysis of the film. The membrane permeability was changed by using variable hydrolysis times. In fact, an electrode prepared by depositing 10 μL of 5% cellulose acetate in a 1:1 acetone/cyclohexanone mixture after 12 h of stirring and evaporation of the solvent worked as follows: non-hydrolysed film excluded most species, and only the small phenol molecules (MW = 94) diffused rapidly through its small pores and gave rise to well-defined peak currents. The permeability of the film increased gradually on increasing the hydrolysis time. Such large species as NADH and ferrocyanide diffused through the film only after hydrolysis for 40 min or longer. Also, hydrolysis times beyond 50 min resulted in coating instability and the film falling apart from the supporting electrode. The hydrolysed film coating formed an effective barrier for large proteins (*e.g.* serum albumin) while allowing diffusion of many solutes of physiological interest. By simultaneously using two electrodes of different permeability, enhanced qualitative information (*e.g.* chromatographic elution peaks) could be acquired similarly as in dual-potential monitoring [25].

Sittampalan and Wilson studied the effect of coating platinum electrodes with cellulose films in comparison with a bare platinum electrode and arrived at the following conclusions: (*a*) the current response of the platinum electrode to hydrogen peroxide was virtually unchanged by exposure to complex environmental samples, as reflected in the identical peak currents obtained—the typical poisoning effect of these matrices was thus seemingly overcome; and (*b*) injections of blank complex matrices gave rise to negligible currents, thus indicating that virtually no electroactive species diffused across the film [26].

One other design developed by Wang's group uses the same base sensor (GCE), which is coated with a layer of poly(4-vinylpyridine) (PVP). This cationic polyelectrolyte was one of the first polymers used to modify electrode surfaces [27]. Much research effort in this context has been directed to the characterization of the transport and electrostatic binding of multi-charged anions at PVP-coated electrodes. The ability of this polymer

to bind counterionic reactants has been exploited for preconcentration of analytes from dilute solutions [27,28]. Lowering the overvoltage for some analytes via electroactive reagents immobilized in PVP is one major analytical advantage of PVP-modified electrodes [29,30]. Based on this evidence, Wang *et al.* built a sensor of this type for use in hydrodynamic systems (HPLC and FIA) by using a straightforward procedure involving immersion of the bare CGE for 30 s in a stirred solution containing 0.4% polymer in ethanol following polishing with albumin and rinsing with bidistilled water. The coat film remaining after the solvent was evaporated was 11 μm thick. The most substantial effect of the protonated PVP film was the enhanced selectivity arising from exclusion of cationic species from the surface. Notwithstanding the increased diffusional resistance resulting from the presence of the membrane, detection limits as low as *ca.* 0.04 ng ascorbic acid and 0.10 ng uric acid were obtained. Protection from organic surfactants was combined with the charge-exclusion effect by using a bilayer coating in the form of a cellulose acetate film atop the PVP layer (deposited by syringing 10 μL of 5% cellulose acetate solution in 1:1 acetone/cyclohexanone on top of the PVP layer, followed by 40-min hydrolysis in a stirred 0.07 M KOH solution). In this way, the sensor performed brilliantly in the determination of the analytes in urine samples [31].

Even though the advantages of using GCE coated with membranes of the perfluorosulphonated polymer Nafion (*e.g.* ion-exchange capacity, high stability and flexibility) have been extensively exploited in the last few years [32–35], and preferential incorporation of organic counter-ions into the polymer was known to further increase the sensitivity and selectivity for trace [33] and *in vivo* measurements [33], respectively, no application to flowing systems had been reported until Wang showed detection in dynamic systems to be substantially enhanced by using electrodes coated with charged Nafion films, which tend to exclude anionic interferents. The enhanced discrimination properties arising from the analyte charge (similar to the size-exclusion effect of cellulose coatings) result in significantly increased selectivity towards the species of interest. The charged coating tends to exclude anionic and neutral interferents, thus adding a new selectivity dimension to electrochemical detection in dynamic systems. A highly selective response was observed for cationic neurotransmitters in the presence of otherwise interfering substances including ascorbic acid, uric acid, bilirubin and chlorpromazine. The low dependence on the flow-rate was found to result in low noise and detection limits of 0.04 and 0.10 ng for

norepinephrine and epinephrine, respectively. Use of an electrode coated with a cellulose acetate film over the Nafion layer provided both enhanced selectivity and protection [36]. Films of Eastman AQ55D (polyester sulphonic acid) cation-exchanger, which are commercially available in dissolved form, were deposited onto GCE surfaces to provide electrochemical sensors of appealing permselectivity, ion-exchange capacity and antifouling properties. Improved selectivity can also be accomplished by excluding anionic species from the electrode surface. This type of sensor has been shown to exhibit its charge-selective behaviour in the presence of a variety of substances of neurological significance. It was found to expeditiously respond to dynamic changes in the concentration of cationic and neutral species and to bind multi-charged counter-ions via the polymer [37].

One of the latest contributions of Wang's group is the joint use of surface-bound redox mediators to enhance electron-transfer kinetics and thus lower the operating potentials of cellulose acetate by incorporating the catalyst (cobalt phthalocyanin, CoPC) into the film domain. In one such structure, the film serves as a template that determines the structure and transport characteristics, while the catalyst functions to enhance the electron-transfer kinetics. With cellulose acetate (CA) films, permeability of the two-domain structure is controlled by basic hydrolysis similarly as in single-domain cellulose films. The mixed CoPC/CA coating thus exhibits properties that surpass those of the two components in isolation, thereby expanding the scope for these electrochemical sensors [38]. Membranes of other materials including Nafion have been used by Wang's group to accommodate the same catalyst in order to develop flow-through sensors that enable effective differentiation between compounds undergoing electrocatalysis from their characteristic charge. The practical analytical use of these sensors was demonstrated by implementing selective flow-injection measurements of hydrazine and hydrogen peroxide in the presence of oxalic and ascorbic acid, respectively. The detection limits thus achieved were quite low (*e.g.* 5.7 ng of hydrazine) [39]. Other mixed coatings such as Eastman AQ polymers plus $\text{Ru}(\text{bipyridyl})_3^{2+}$ have been used to construct sensors of this type and confirm the synergistic effect of the two components when used in conjunction [37].

The peculiar electrochemical behaviour of electrode surfaces coated with a layer of phosphatidylcholine (PC) adds a new dimension to the practical applications of this type of sensor as it enables development of electrochemical processes within a lipid layer. Redox-active amphiphiles readily

partition into the PC lipid layer from a contacting aqueous solution and undergo electrochemical reactions at the sensor surface. By contrast, more hydrophilic variants of these electroactive compounds are excluded by the lipid layer, which thus prevents their electron-transfer reactions with the sensor. Several research groups are actively investigating the properties of electrode surfaces derivatized with hydrophobic materials. One approach commonly used in this context involves self-assembling of electroinactive [40–44] or electroactive amphiphiles [45–51] at the electrode/solution interface. One alternative approach uses the Langmuir–Blodgett technique to transfer monolayers [46,52–57] or multi-layers [58] from the air/solution interface to a substrate that is then employed as an electrode. The effects of cast layers of palmitic acid on the electrochemical behaviour of various substances at the underlying electrode were recently discussed by Tanaka [59], who showed hydrophobic layers to considerably hinder observation of the electrochemistry of hydrophilic species. Preparing this type of sensor is very simple as it only entails depositing an accurate volume of modifier solution on the tip of the electrode (usually a GCE) and allowing the solvent to evaporate. Redox-active amphiphiles are extracted from the contacting aqueous solution into the cast lipid layer, where they undergo electrochemical reaction with the underlying electrode surface. Once the lipid layer is loaded with the electroactive amphiphile, the electrode can be transferred to solutions of pure supporting electrolyte while retaining electroactivity. On the other hand, hydrophilic electroactive substrates are kept away from the electrode by the modifier layer, thus largely preventing observation of electron-transfer reactions with the electrode [60].

4.3.2 Sensors based on integrated liquid–liquid extraction and detection

Integrating liquid–liquid extraction and detection is far from easy, as reflected in the few attempts made so far. Many of the devices developed for this purpose fail to comply with the definition of sensor. Such is the case with continuous liquid–liquid extraction systems without phase separation, where programmed switching of the propulsion system (a peristaltic pump) allows the extracting phase to be passed iteratively by the detection point in a back-and-forth motion that enriches it gradually with the extracted species [9–11]. This type of system is much too complex to be considered a sensor, though; in addition, the extraction process is not completely simultaneous with detection.

The device proposed by Pawliszyn, based on the establishment of a concentration gradient, complies more strictly with the definition of sensor. However, it can only be used with detection techniques capable of probing the typically small volumes associated with diffusion layers, which are most

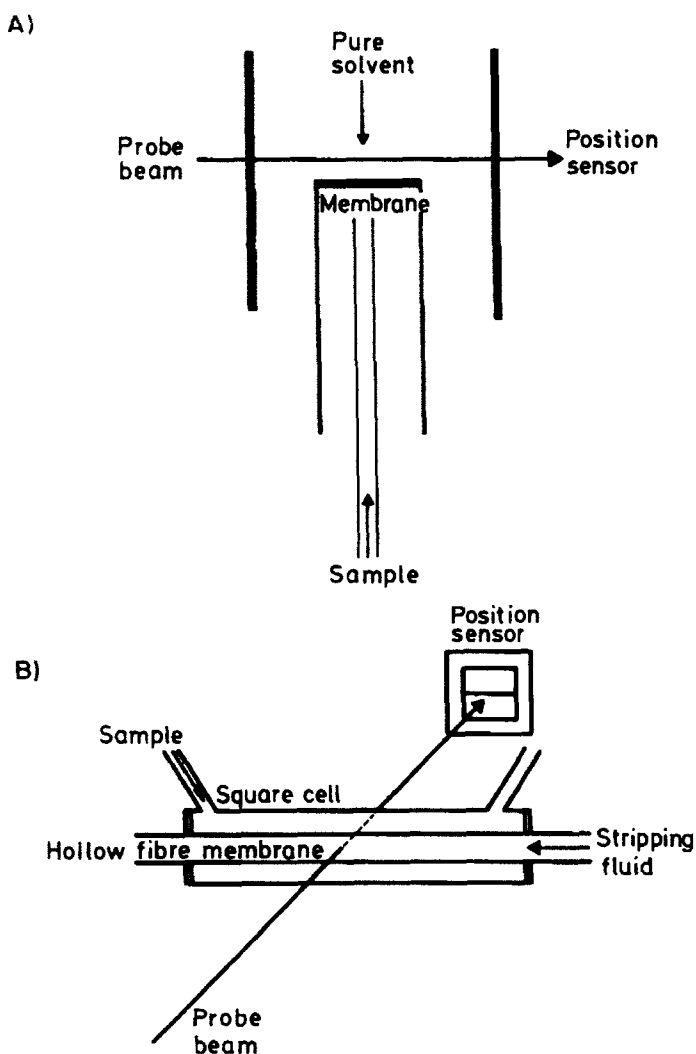


Figure 4.3 — Experimental designs using a planar porous membrane (A) and a silicone hollow fibre membrane (B) for the implementation of sensors based on integrated liquid-liquid extraction and detection. (Reproduced from [61] with permission of the American Chemical Society).

often lower than 100 μm . Figure 4.3 shows the two designs proposed by Pawliszyn. One of them (Fig. 4.3.A) consists of a cellulose nitrate membrane of 0.2 μm pore size that is glued carefully onto one end of a piece of 2 mm OD \times *ca.* 1 mm ID glass tubing. The capillary is inserted into a 5-cm length of 3-mm ID tubing and aqueous samples introduced into the system via a 0.5-mm OD fused silica capillary. The pure solvent (water) is delivered by means of microtubing made of polyfluorocarbon resin. The other design (Fig. 4.3.B) consists of a single silicone hollow fibre membrane of 0.3 mm ID and *ca.* 0.6 mm OD placed in a 2-cm long \times 1 mm ID piece of square tubing. The stripping fluid (hexane or water) flows through the centre of the fibre while the aqueous samples flow around the outside of the fibre and enter the system via a fused silica capillary. The diffusion modules are attached to a vertical translation stage to allow precise focussing of the laser beam onto the membrane surface. The concentration gradients formed during the extraction of analytes from an aqueous to an organic phase can be quantified via the refractive index gradient by measuring the deflection of the focussed laser beam that passes near the interface between the two phases. The maximum of the concentration gradient transient is proportional to the analyte concentration in the sample. The steep concentration gradients created at the interface at the initial stages of the mass transport process ensure good sensitivity. The detection limit of this technique is proportional to the distance of the probing laser beam from the interface and the diffusion coefficient of the analyte in the organic phase. The sensitivity of this special type of sensor is related to other properties of the organic phase as described by the distribution constant. In addition, separation of species with substantially different diffusion coefficients is fairly easy to accomplish since the time corresponding to the maximum of the transient is inversely proportional to the diffusion coefficient. The sensor enables very rapid analytical determinations since quantitation is performed at the initial stages of sample preparation [61].

4.4 INTEGRATED RETENTION AND DETECTION

Most flow-through sensors integrating retention and detection involve placement of an inert support in the flow-cell of a non-destructive spectroscopic detector where the analytes or their retention products are retained temporarily for sensing, and then eluted. Rendering these sensors reusable entails including a regeneration step suited to the way retention is performed.

In the best of cases, the carrier itself acts as regenerator; otherwise, a continuous configuration (usually an FI manifold) is the most convenient choice for this purpose.

The equipment required to develop this type of sensor is very simple and resembles closely that used to implement ordinary liquid–solid separations in FI manifolds. The only difference lies in the replacement of the packed reactor located in the transport-reaction zone with a packed (usually photometric or fluorimetric) flow-cell accommodated in the detector. Whether the packing material is inert or active, it should meet the following requirements: (a) its particle diameter should be large enough ($< 80\text{--}100\ \mu\text{m}$) to avoid overpressure; (b) it should be made of a material compatible with the nature of the integrated detection system (*e.g.* almost transparent for absorbance measurements); and, (c) the retention/elution process should be fast enough to avoid kinetic problems.

Although somewhat specialized, integrated flow-cells are normally commercially available. Ideally, they should be short (0.2–1.5 mm) and narrow-bore in order to avoid problems arising from inadequate detector capacity and sensitivity, respectively. Ideally, they should also have small inner volumes in order to boost sensitivity and sample throughput.

A logical classification of the sensors dealt with in this section is based on which species (the analyte or a reaction product) is retained and monitored. However, because sensors based on retention of the analyte and measurement of some intrinsic property are few and far between, adopting such a classification here would be rather impractical. On the other hand, because most of these sensors—those based on mass measurements excluded—rely on retention of the product of a prior chemical reaction, a classification based on the type of support or retention used would also be inappropriate since the material or phenomenon involved in the retention process logically varies with the ionic or molecular species formed. Because sensors based on retention and detection of the analyte itself are the simplest, they are discussed first in each section, which deals with a different type of detector.

It should be noted that, if a prior chemical reaction is involved, the process taking place at the cell cannot be merely retention/detection since the separation alters the reaction equilibrium, so it also takes place to some extent in the sensing zone.

4.4.1 Sensors based on integrated retention and luminescence detection

Fluorimetry excels photometry for detecting analytes and products by means of sensors on account of its higher sensitivity and the also higher selectivity arising from the small number of species that can emit previously absorbed energy in a radiant form. As a rule, the fluorescence of an analyte or reaction product increases on retention on a solid support as a result of losses

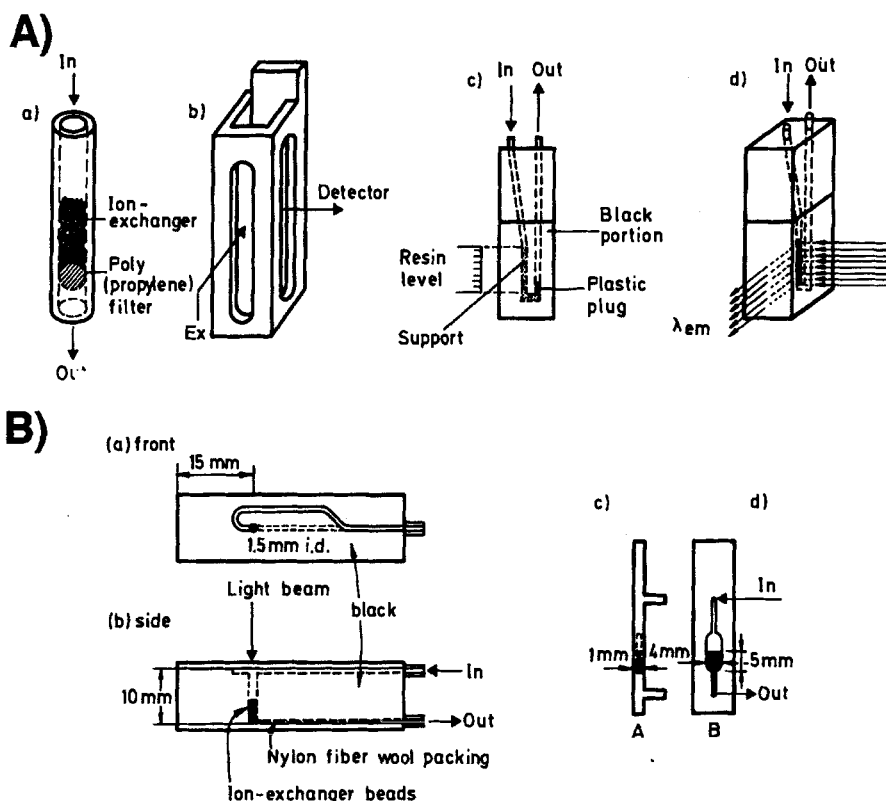


Figure 4.4 — (A) Flow-through cells for spectrofluorimetric sensors: (a) fused silica tube (1.5 mm ID) packed with 1 mg of CM-Sephadex C-25; (b) micro-cell holder; (c) side and (d) front view of a commercially available sensor. (Reproduced from [62] and [64] with permission of the Royal Society of Chemistry and Elsevier Science Publishers, respectively). (B) Flow-through cells for photometric sensors. Side and front views of two commercially available designs. For details, see text. (Reproduced from [80] and [83] with permission of Elsevier Science Publishers and the Royal Society of Chemistry, respectively).

of absorbed energy being minimized by the enhanced rigidity of the retained species in its bound form. The sensitivity of this type of sensor is limited by (a) the luminescence of the support packed in the flow-cell, which reduces the cell thickness and hence the amount of monitored species that can be retained at the detection point; and (b) the detector being reached by radiation dispersed by particles of the solid support.

A fluorimetric sensor based on the native fluorescence of some alkaline earth metals using a small fused silica tube of 1.5 mm ID and 4 mm OD packed with CM-Sephadex resin (Figs. 4.4.A.a and 4.4.A.b) was employed for the determination of europium, terbium, dysprosium and samarium from the fluorescence band resulting from $d \rightarrow f$ electronic transitions, which are enhanced by sorption of these elements in a weakly acidic cation exchanger. The sensitivity achieved was found to depend on both the injected sample volume and the native fluorescence of the analyte. Thus, a detection limit of 22 ng of europium was achieved by using 8.3 mL of sample. The sensor was made reusable by eluting the target element with a solution of nitric acid injected via a valve positioned serially with the analyte injection valve in a single-channel manifold including the sensor (Fig. 4.5) [62].

A similar configuration was used by Cañizares *et al.* to implement a sensor for aluminium based on the formation of a fluorescent complex between the analyte and salicylaldehyde picolinoylhydrazone. The sensor was built from a commercially available fluorimetric flow-cell (Hellma 178.12QS, 1.5 mm pathlength) that was packed with C_{18} bonded silica beads and placed in an ordinary fluorimeter. The complex, formed along the manifold on merging the injected sample with a reagent stream (Fig. 4.5), was retained in the sensor, measured, and finally eluted with 200 μ L of 2 M HCl injected near the detector. The method thus implemented features a linear determination range from 2 to 200 ng Al/mL, good repeatability and excellent selectivity [63]. However, the ideal continuous configuration for implementation of sensors based on retention of the product of a prior reaction is one of symmetric merging zones (see Fig. 2.12.4), with or without a switching or injection valve located after the sample and reagent insertion valve. The need for one such auxiliary valve is dictated by whether or not an eluent other than the carrier is required. This type of configuration and fluorescent complex-formation reaction has been used to implement various sensors for anions and B_6 vitamins.

The first such sensor, developed for the determination of fluoride ion, relies on the formation of the zirconium(IV)–Calcein Blue–fluoride complex

and its retention in an ionic resin (DEAE–Sephadex) packed in a commercially available flow-cell such as that shown in Figs 4.4.A.c and 4.4.A.d (a Hellma 178.12QS model) [64]. The sample and the zirconium–Calcein Blue binary complex are simultaneously injected into a water and a 5×10^{-3} M HCl stream, respectively, which form the fluorescent species on merging prior to the detector; there, the ternary complex is retained for the time needed to acquire the analytical signal and subsequently eluted by injecting 850 μ L of 0.4 M HCl via a third valve located near the detector. This sensor exemplifies the advantages of flow-through sensors over conventional, probe-type sensors. Table 4.1 compares the typical parameters for the flow-through sensor and an earlier probe-type sensor [65] based on the same chemical reaction. As can be seen, the former clearly surpasses the latter in terms of sensitivity, reproducibility, service life and sample throughput. Also, the selectivity is much higher than that of the manual method based on the same reaction [66].

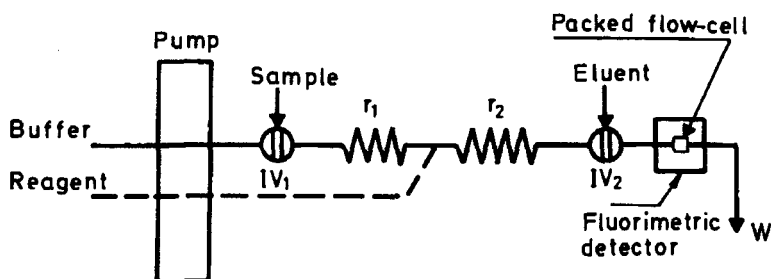


Figure 4.5 — Generic manifold for implementation of methods using fluorimetric sensors for directly measuring the intrinsic fluorescence of the analyte (single-channel system) or a reaction product (by including the zone bound by the dotted line for reagent supply).

The cyanide sensor developed by the authors' group is based on the formation of an addition product between cyanide ion and pyridoxal-5-phosphate, and its subsequent retention in the sensor (a fluorimetric flow-cell packed with QAE–Sephadex resin). The eluent is not injected, but merged with a stream of 0.05 M HCl after the reactor that is used both to acidify the complex and elute it after measurement. The calibration graph for the target analyte was linear from 50 ng/mL to 3.0 μ g/mL, and the relative standard deviation and sample throughput were 1.4% (for 2 μ g CN[−]/mL) and

10 samples/h, respectively. The selectivity of this sensor is clearly higher than that of conventional continuous methods, both fluorimetric and photometric [67].

Table 4.1. Comparison of the performance of a probe-type and a flow-cell type sensor for fluoride

Parameter	Probe-type sensor	Flow-cell type sensor
Determination range	0.5–8 $\mu\text{g/mL}$	1–40 ng/mL
Detection limit	0.5 $\mu\text{g/mL}$	1 ng/mL
Time for:		
Preparation	Overnight	< 15 min
Measurement	30 min	1 min
Regeneration	60 min	1 min
Reproducibility (RSD)	15%	1%
Sensor lifetime	15 measurements	> 100 measurements

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The fluorescence of pyridoxal is dramatically increased by complexation with beryllium ion, which provides a means for accurate measurements of these B_6 vitamers by using a flow-cell packed with C_{18} bonded silica beads. The complex is eluted from the sensor by the aqueous stream itself, into which the sample and beryllium are simultaneously injected [68].

Notwithstanding the excellent analytical features inherent in molecular phosphorimetric measurements, their use has been impeded by the need for cumbersome cryogenic temperature techniques. The ability to stabilize the "triplet state" at room temperature by immobilization of the phosphor on a solid support [69,70] or in a liquid solution using an "ordered medium" [71] has opened new avenues for phosphorescence studies and analytical phosphorimetry. Room-temperature phosphorescence (RTP) has so far been used for the determination of trace amounts of many organic compounds of biochemical interest [69,72]. Retention of the phosphorescent species on a solid support housed in a flow-cell is an excellent way of "anchoring" it in order to avoid radiationless deactivation. A configuration such as that shown in Fig. 2.13.4 was used to implement a sensor based on this principle in order to determine aluminium in clinical samples (dialysis fluids and concen-

trates). The chemical foundation of the sensor lies in the formation of a complex between aluminium and the phosphorogenic reagent ferron (8-hydroxy-7-iodo-5-quinolinesulphonic acid). The analytical figures of merit of the sensor are as follows: detection limit 2 ng Al/mL; precision (as RSD) for 40 ng Al/mL, 3.2%. Also, none of the ions commonly found in biological samples was found to interfere seriously except Fe(III), which was masked by addition of 1,10-phenanthroline. Basic experiments on the properties of the RTP exhibited by various media used to ensure the required retention rigidity (micelles, vesicles, filter paper, strong anionic resins, *etc.*) showed Dowex 1-X2-100 resin to provide the best sheltering for the excited triplet state [73].

4.4.2 Sensors based on integrated retention and photometric detection

Solid-phase absorptiometry, extensively developed by Yoshimura *et al.* [74], is the most immediate predecessor of the sensing approaches discussed in this section. The batch methods reported by these Japanese authors are extremely time- and labour-consuming as they involve reaction development, retention on support particles immersed in the solution, filtration and washing of the particles, placement in the measuring cell and unloading and washing of the cell after sensing. The support is not regenerated, which results in extremely high costs and, usually, inapplicability to routine analyses. Nukatsuka *et al.* [75] developed a system lying between batch and flow-through sensors based on retention and photometric detection for the determination of beryllium. For this purpose, they had the analyte react with Chromazurol B (CAB) to form a complex that was bound to a cellulose membrane filter by vacuum filtration. After excess CAB was removed by washing the filter, the membrane showed a coloured circle, the absorbance of which at 615 nm was immediately measured by using a moist clean filter.

One of the few existing flow-through sensors based on direct measurements of the analyte absorption was developed by Yoshimura *et al.* for the determination of copper [76]. They used a single-channel manifold including two serially arranged injection valves for the sample and a regenerating nitric acid solution in addition to a conventional photometer. The sensor consisted of a commercially available flow-cell (Fig. 4.5.B) that was packed to an appropriate height with Bio-Rad AG50W-X12 resin of 100–200 mesh in hydrogen form. The photometer was placed vertically in order to make the top part of the ion-exchange material flat. The calibration graph run was linear over the range 0.01–0.5 μmol copper.

The increased selectivity derived from *in situ* concentration of the retained material used to pack the flow-cell can be furthered by inserting an ion-exchange column into the FI manifold in order to implement a pre-concentration step. Such is the case with the sensor proposed by Yoshimura *et al.* for the determination of bismuth traces. The manifold effects a prior preconcentration of the analyte as a chloro complex by means of a column packed with Dowex 1-X8 anion-exchange resin. The complex is desorbed with 0.15 M sulphuric acid and subsequently mixed with a stream of 1 M KI that drives it to the detector, containing QAE A-25 anion exchanger, for measurement of the iodine complex formed in the way at 472 nm [77]. Yoshimura's group have used other flow-through sensors and an FI configuration similar to that of Fig. 2.12.4, in addition to an upright photometer and a flow-cell such as that depicted in Figs 4.4.B.a and 4.4.B.b, packed with a suitable support, for the determination of chromium by monitoring the product of its reaction with 1,5-diphenylcarbazine [78]; that of iron by use of 4,7-diphenyl-1,10-phenanthrolinedisulphonate [79]; that of molybdenum with Tiron as reagent [80]; and those of phosphate [81] and silicic acid [82] using molybdate and Malachite green in both cases. The sensitivity, selectivity and precision were quite good in all instances.

A flow-through sensor for ammonia based on retention of the product of the Berthelot reaction on a Sephadex QAR support packed in a flow-cell such as that shown in Figs 4.4.B.c and 4.4.B.d was developed by the authors' group. The retained product was eluted after measurement by means of a cationic surfactant included in the carrier solution. In this way, ammonia was determined at concentrations between 0.4 and 20.0 $\mu\text{g/mL}$ with an RSD of 0.8% and a throughput of 13 samples/h. The determination limit of the sensor was lowered by a factor of 10 by using a flow-cell twice as long, but the throughput was also reduced as result. Application of the sensor to the determination of ammonia in agricultural samples (plants and soils) provided results comparable to those obtained by using the recommended methods for these types of sample [83].

The determination of formaldehyde, a substance of great interest on account of its toxicity and high significance to the resin, fertilizer and explosive manufacturing industries, among others, entails the use of rapid, convenient, safe methods. One sensor developed for this purpose uses the manifold shown in Fig. 4.6 to handle the reagents involved, *viz.* *p*-rosaniline (PRA) and sulphite. The instability of sulphite ion calls for on-line mixing with PRA dissolved in 0.4 M HCl prior to injection of the sample. After the

reaction completes along reactor R, the resulting product is retained by Dowex 1-X8 anion-exchange resin packed in an OS 138 flow-cell (Figs 4.4.B.c and 4.4.B.d), from which it is eluted with 2 M HCl after the analytical signal is recorded. Figure 4.6 also shows the record obtained and the valve switching times used. The selectivity thus achieved is quite good (detection limit $0.3 \mu\text{g/mL}$ and 0.75 ng/mL for 1 and 2 mL of sample, respectively), and so is the precision (2.8% and 1.3% for 2 and $20 \mu\text{g/mL}$ formaldehyde, respectively). The throughput varies with the working conditions and ranges between 10 and 18 samples/h. The sensor provided excellent results in the determination of formaldehyde in well water [84].

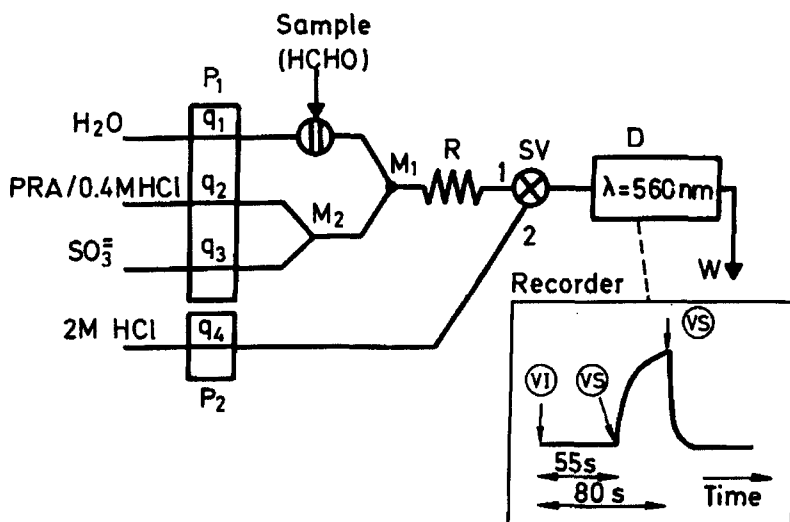


Figure 4.6 — Manifold used and recording obtained with the flow-through photometric sensor for the determination of formaldehyde. PRA *p*-rosaniline; q flow-rate; P peristaltic pump; M mixing point; R reactor; SV switching valve; IV injection valve; D detector; W waste. (Reproduced from [89] with permission of Marcel Dekker, Inc.).

Ever since Albrecht and Creighton [85], and Jenmarie and van Duyne [86], observed that the Raman cross-section for pyridine absorbed on a roughened silver electrode was larger than that in solution by six orders of magnitude, surface enhanced Raman spectroscopy (SERS) has steadily gained ground in analytical instrumentation. The sensitivity of this technique

is comparable to that of conventional fluorescence absorption spectroscopy, yet its selectivity is much higher as a result of the inherent discriminating power of vibrational spectroscopies [87,88]. Not all the supports tested in this context (silver electrodes, sols, films, island coatings, thin-layer chromatographic plates, filter paper, silver-coated spheres and prolate silica posts, among others) are appropriate for constructing a sensor; however, future developments may allow them to be unrestrictedly incorporated into sensors. The results obtained by Winefordner *et al.* using silver hydrosols and silver-coated filter paper are very promising [89].

4.4.3 Sensors based on integrated retention and mass detection

Several flow-through sensors using a piezoelectric quartz balance to house a (bio)chemical reaction were discussed in Chapter 3 in relation to the determination of both gaseous and dissolved species. The changes detected by this type of sensor can also arise from adsorption of the analytes on the active surface coating. This principle has been used to develop mass sensors for the determination of gases and dissolved ionic analytes, as well as monitoring biotechnological processes with the aid of microbial biofilms formed on the sensing surface, among others.

Slater *et al.* [90] studied the response of the conducting polymer poly(pyrrole) to selected gases and vapours by using two different procedures involving measurement of resistance and mass changes with the aid of piezoelectric quartz microbalances in order to characterize responses for incorporation into sensor arrays. They exposed bromide-doped films to methanol, and *n*-hexane, 2,2-dimethylbutane, ammonia and hydrogen sulphide, as well as polymer films of variable thickness, to methanol vapours in order to analyse the response profiles obtained. This latter approach was the more promising. Absorption of methanol into poly(pyrrole) appears to be a two-stage process involving first the penetration of vapour into the polymer, accompanied by swelling, and then diffusion at a high rate into the swollen rubbery material. Full recovery could not be obtained in a single exposure, a second exposure of a modified polymer containing initial concentrations of methanol. Because swelling was the rate-determining step of the sorption, subsequent exposures would take the polymer to the same point in the sorption curves as the first 5-min exposure. Following a systematic study involving various gases and vapours, the authors concluded that the response mechanism of poly(pyrrole) sensing of these species was a combination of electronic and physical effects. They concluded that

development of reliable mass sensors for gases based on the use of these materials required deeper research. Krawczynski *et al.* [91] expanded the number of materials and scope tested by studying such crystal-coating materials as pyridoxine, glutamic acid, histidine, methionine, alanine and cysteine (all in hydrochloride form) with a view to developing sensors for ammonia. The first three coating materials allowed the analyte to be determined at concentrations from 1 ng/mL to 1000 µg/mL and provided sensors that were serviceable for 1–4 months. Other coating materials used in the determination of SO₂ provided very favourable results [91].

Electrochemical quartz-crystal microbalances (EQCMs) continue to be used intensively for monitoring the exchange of mobile species between a polymer-modified electrode and its bathing solution. Even though the universality of mass detection and the high sensitivity of EQCMs are two major advantages, the selectivity needed in many determinations entails separation of the total mass change into single-species components, which is extremely complex [92].

The biochemical applications of this type of sensor include the determination of adenosine 5'-phosphosulphate reductase and human chorionic gonadotropin [93], as well as the indirect quantitation of microorganisms in a liquid phase by monitoring metabolite production [94]. More recently, Nivens *et al.* used a quartz crystal microbalance to monitor the formation of *Pseudomonas cepacia* biofilms in a non-destructive fashion. The experiments involved long-term monitoring (a few days), which posed problems arising from baseline drifts not encountered in short-term experiments or those carried out in the air or *in vacuo*. Some true flow-through sensors based on integrated retention and mass detection have been constructed from 5-MHz Ar-cut quartz crystals sealed into flow-cells and exposed to aqueous environments. Under these conditions, the sensors are more responsive to pressure and temperature changes than when the crystals are exposed to the air. The problems derived from both the special flow-through sensor used and long-term monitoring were circumvented by writing a program to control data acquisition via a general-purpose interface bus and the program Ayst was used to convert frequency shift data into cell count data, differentiate the latter and smooth the differentiated data using a low-pass Blackman filter with a cut-off frequency of 1 h⁻¹. The results obtained showed the sensor to be suitable for monitoring sterile aquatic environments for a few days and an extremely sensitive device for detecting microbial contamination in ultrapure water. In addition, the results were consistent with

available knowledge of biofilm formation, so the sensor can also be used to study this type of process. Current research aimed at expanding its scope of application involves studying the effect of varying the surface and liquid-phase chemistry on biofilm formation and applying temperature and pressure compensation methods [95].

4.5 FLOW-THROUGH SENSORS FOR MULTIDETERMINATIONS BASED ON INTEGRATED RETENTION AND DETECTION

Sensors allowing two or more analytes to be determined by a simple procedure are technically more complex than single-parameter sensors, but also more advantageous, which justifies the R&D endeavours devoted to them. Few multi-parameter sensors have so far been developed, however, and even fewer are actually operative, of which those commercially available are included. Progress in this area is therefore much needed and desired. Multi-determinations based on sensors can be accomplished (a) by using a detector capable of distinguishing the signal produced by each analyte, whether as

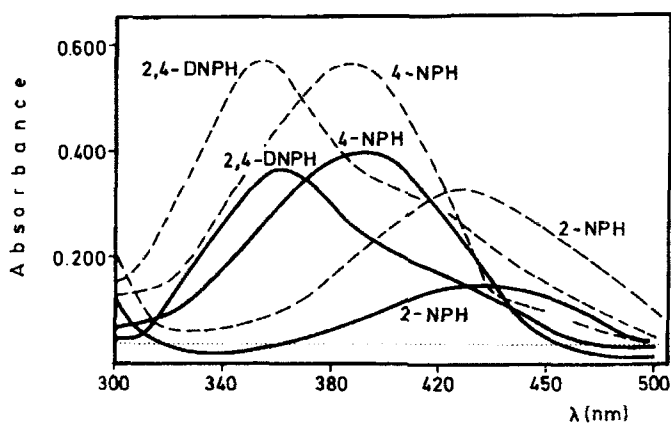


Figure 4.7 — Absorption spectra of 2,4-dinitrophenylhydrazine (2,4-DNPH), 4-nitrophenylhydrazine (4-NPH) and 2-nitrophenylhydrazine (2-NPH) in solution (solid line) and retained on C_{18} bonded silica of 60-100 μm particle size (broken line). The dotted line corresponds to the blank spectrum in the second type of experiment (cell packed with C_{18} bonded silica in distilled water). (Reproduced from [97] with permission of Elsevier Science Publishers).

such or on subjection to a chemical reaction; and (b) by separating the analytes using an on-line coupled chromatographic or non-chromatographic technique, aided or not by a prior chemical reaction. Depending on the particular approach used, multi-determinations can be simultaneous (which in FIA terms means that two or more analytes are determined in a single injection operation [96]), or sequential (each analyte requires one injection).

4.5.1 Multi-sensors based on integrated retention and discriminating detection

The ability of diode array spectrophotometers to monitor in a simultaneous manner the absorbance at several wavelengths allows one to select specific λ values where the behaviour of the analytes (or their reaction products) is sufficiently different for discrimination purposes. Thus, the authors' group developed a method for the resolution of mixtures of amines (2,4-dinitrophenylhydrazine, 2-nitrophenylhydrazine and 4-nitrophenylhydrazine) based on (a) the use of a single-channel hydrodynamic system and (b) a flow-cell packed with a suitable material (C_{18} resin); (c) placement of the flow-cell in a diode array spectrophotometer; and (d) acquisition and processing of the intrinsic absorbance data for the analytes at the selected measuring wavelengths. Figure 4.7 shows the absorption spectra of the target analytes; as can be seen, they were extensively overlapped, so they could only be distinguished by careful selection of the wavelengths at which the individual spectra were most markedly different from one another. The experimental procedure was as follows: the sample containing the analytes was injected into a stream of 6:4 v/v phosphate/methanol buffer acting as carrier and eluent. Because no chemical reaction was needed prior to detection, the distance between the injection and detection point was minimal, which resulted in a short residence time. The fast kinetics of retention/elution allowed a determination limit of 5×10^{-7} M to be achieved. By solving the 10-equation system obtained from measurements made at 10 different wavelengths, mixtures of these amines were resolved with fairly small errors [97].

A photometric flow-through sensor for the determination of carbamate pesticides (carbofuran, propoxur and carbaryl) based on similar principles as regards the detector and sensor used (a diode array spectrophotometer and a flow-cell packed with C_{18} resin, respectively) was employed to monitor the formation of the products resulting from hydrolysis of the analytes and on-line coupling of the respective phenols with diazotized sulphanilic acid. This

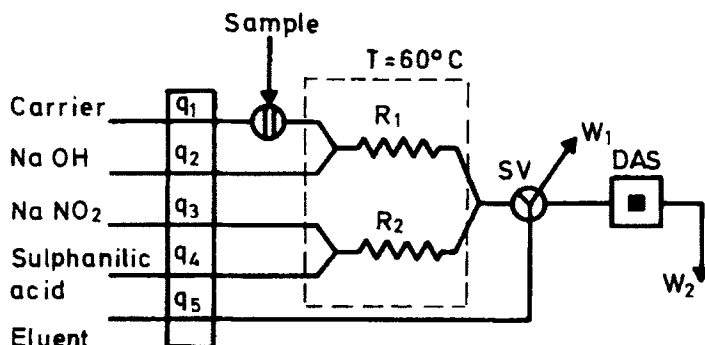


Figure 4.8 — Manifold used for implementation of the flow-through sensor developed for the determination of carbamate compounds based on hydrolysis of the analytes and dye formation. q flow-rate; R reactor; SV switching valve; DAS diode array spectrophotometer; W waste. (Reproduced from [98] with permission of the American Chemical Society).

preliminary reaction was implemented in an FI manifold such as that depicted in Fig. 4.8, where the sample was injected into an aqueous carrier and merged with a basic stream to hydrolyse the analytes along reactor R_1 . On mixing of the nitrite and sulphanilic acid streams, the diazotation reaction took place in R_2 , followed by the dye formation on merging with R_1 prior to the flow-cell, where the dye was retained and detected. After absorbance–data pairs were acquired at the nine selected wavelengths, the actuating valve SV drove a stream of 1:1 ethanol/2 M HNO_3 to the sensor, from which the retained products were rapidly flushed. The throughput thus achieved was 40 samples/h and the determination range encompassed concentrations in the nanogram to microgram per millilitre range. The sensor was used to resolve mixtures of the target analytes in various types of water with excellent results. The enhanced sensitivity arising from *in situ* concentration was apparent in a comparison of the determination limits obtained with those afforded by a conventional FIA method based on the same continuous configuration but including no packing in the flow-cell (the former were up to 50 times lower than the latter) [98].

Derivative synchronous fluorescence spectrometry is a useful technique for the simultaneous determination of related species in mixtures with no prior separation as it combines the assets of derivative [99] and synchronous

techniques [100,101]. It has so far proved useful for resolving a variety of mixtures [102]. Whenever the resolving power of this spectrometric technique is inadequate for the intended purpose, chemical manipulation of the sample may facilitate the multi-determination provided the derivatizing reaction used is selective under given conditions. Both effects were combined in a multisensor for the determination of B₆ vitamers (pyridoxal, pyridoxal 5-phosphate and pyridoxic acid). This sensor multidetermination relies on (a) formation of fluorescent derivatives of the analytes with beryllium in an ammonia buffer at various pH values; (b) use of a flow-cell packed with C₁₈ bonded silica beads; (c) synchronous recording of the fluorescence of the retained species after the flow is stopped; and (e) spectral derivatization to resolve the mixtures. The experimental procedure used for this purpose is as follows: sample solutions and the buffered beryllium solution are injected simultaneously via a dual valve and carried by distilled water (Fig. 4.9). Each sample solution is injected twice simultaneously with the beryllium–buffer solution at pH 7.9 or 9.9. The reactions products formed along a 3-m reactor are trapped by the C₁₈ material. The flow is stopped 2 min after injection and a synchronous scan is performed at a 5-nm difference between the emission and excitation wavelengths from 500 to 320 nm (emission) at a rate of 180 nm/min. The synchronous scan is then converted into its first

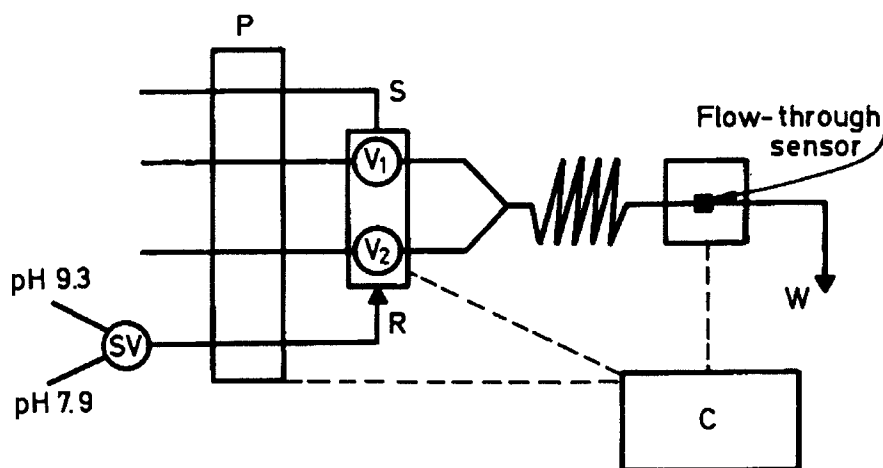


Figure 4.9 — Merging-zones manifold coupled to a flow-through sensor for the determination of B₆ vitamers. P peristaltic pump; SV switching valve; V₁, V₂ injection valves; S sample; R reagent; C microcomputer; W waste. (Reproduced from [103] with permission of Elsevier Science Publishers).

derivative by means of a microcomputer. After the scan, the peristaltic pump is switched back to flush the sensor and make it ready for the next sample. The strong overlap between the spectra for the complexes of pyridoxal and pyridoxal 5-phosphate at pH 9.9, which precludes their resolution, is avoided at pH 7.9, where the pyridoxal complex does not exist. The complex formed by pyridoxic acid poses no problem for resolution. Calibration curves were linear between 50 and 1500 nM (*i.e.* wider than those of the conventional FI method by two orders of magnitude), and the RSD was less than 4.5%. The sensor was used for the determination of the analytes in serum samples with good results [103].

4.5.2 Multi-sensors based on separation followed by integrated retention and detection

A prior separation can be used to facilitate the sequential arrival of several species at the sensor and hence their temporal discrimination, which can be accomplished in two ways, namely (*a*) by removing each analyte or product before the next arrives (the detector response corresponds to a single species in each case), and (*b*) by allowing the analytes or products to accumulate (thereby obtaining a step-line response) and removing them as a whole after measurement. The species to be measured in both cases are similar and exhibit signals at the same instrumental settings. Multi-parameter measurements in turn can be either simultaneous or sequential. The former are performed in a single injection. This operational mode usually calls for a high resolving power (*e.g.* that of a chromatograph). On the other hand, sequential measurements require individual injections (one per analyte).

Tena *et al.* used an HPLC/post-column derivatizing–integrated reaction/detection sensor system for the determination of carbaryl and its hydrolysis product based on separation of the two analytes with the aid of an ultrabasic C₁₈ chromatographic column and derivatization prior to retention in the sensor, in addition to formation of a dye with sulphanilic acid (previously diazotized on-line), which followed hydrolysis of carbaryl. After measurement, the products were eluted by using a stream of 1:1 v/v ethanol/2 M HNO₃ that was driven to the detector by actuating a switching valve near the detector. The method thus developed allows the determination of the two analytes at concentrations between 5 and 800 ng/mL, with an RSD less than 4% and excellent selectivity against other carbamate pesticides [104].

If the discriminating power of the separation system concerned is inadequate, chemical derivatization is one possible aid. Such is the case with a

sensor for speciation of aluminium based on the Driscoll method [105], which allowed the different forms of the metal to be distinguished on the basis of the above-described fluorescent complex-formation with a hydrazine. Figure 4.10 shows the FI manifold used in conjunction with the sensor. A valve located prior to the injection port allows switching between two sample pH values in order to determine total reactive aluminium (pH 1.0) or total monomeric aluminium (pH 3.5). Valve IV_2 is kept in its load position in both determinations, so plugs injected via IV_1 do not pass through the exchange column. A third injection with IV_2 in its unload position allows the sample plug to pass through the column in order to retain charged species and determine non-labile monomeric aluminium. The column is recycled upstream by switching valve IV_2 to its load position. In this way, more efficient regeneration is achieved and gradually increased compactness in the packing material—which usually results in flow-rate oscillations and hence irreproducibility—is avoided. In addition to the three species determined in the three injections, labile monomeric aluminium can be quantified as the difference between total monomeric and non-labile monomeric metal. The sensor is regenerated after each determination and thus made ready for the next one by switching injection valve IV_3 to insert 200 μL of 2 M HCl [106].

4.6 ION-SELECTIVE ELECTRODES (ISEs) AND ION-SENSITIVE FIELD-EFFECT TRANSISTORS (ISFETs)

Even though the first electrochemical sensors based on potentiometric principles were developed early this century (*e.g.* the glass electrode for pH

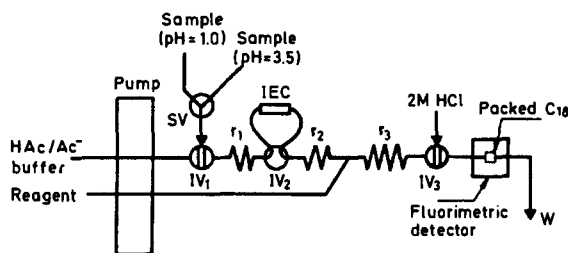


Figure 4.10 — Flow-through sensor for aluminium speciation coupled to an FI manifold for implementation of the Driscoll method. SV switching valve; IV

measurements was devised by Cremer in 1906 [107]), the most dramatic advances in this field have been reported in the last 20 years and involve both ion-selective electrodes (ISEs) and ion-sensitive field-effect transistors (ISFETs). The advantages derived from their use in hydrodynamic systems (particularly FI systems), as demonstrated by Cammann [86], are as follows:

(a) The hydrodynamics in front of the sensing electrode surface responsible for the thickness of the Nernst diffusion layer, which itself determines the response time, are controlled and stable. This results in a high reproducibility in the potentials created.

(b) The liquid junction potential at the diaphragm of the reference electrode is also stabilized by the controlled hydrodynamics and can be further reduced by using an appropriate carrier electrolyte solution, which also leads to more reproducible signals.

(c) Occasionally, the selectivity of an ISE can be dramatically improved as a result of the time during which the sample plug passes over the sensor surface being too short for the full equilibrium potential of interfering ions to be reached. Cammann and Ilcheva [109,110] reported a selectivity improvement of several orders of magnitude for Br^- and I^- as interferences on a single-crystal AgCl electrode. The effect, however, is less pronounced with PVC membrane electrodes [111].

(d) Contamination problems encountered in ordinary "beaker determinations", where the salt bridge solution leaks into the sample via the highly diffusible sleeve diaphragms usually employed, can be circumvented by placing the reference electrode downstream.

(e) The carrier solution circulated along the FI manifold can be specially optimized for ISE applications in order to serve the following additional functions:

- (1) If the carrier solution is identical to that used in the salt bridge of the reference electrode, the liquid junction potential is minimized, and so are its fluctuations.
- (2) A steady flow of solution past the ISE sensing surface helps keep it clean. Deposition of surface layers, which results in deterioration, can be avoided by adding some special species including chelates to prevent salt precipitations, and heparin or trypsin-HCl to hinder the formation of biolayers in analysing biological fluids.
- (3) If the ion to be sensed is present in the carrier solution, the ISE membrane is automatically conditioned by the ion, which

improves responsiveness and diminishes selectivity losses with time. Baseline stability is much higher within the limits of the detection range. In addition, the concentration of the sensed ion in the carrier solution can be adjusted to a limiting value that must be controlled by analysis (environmental monitoring). This allows convenient obtainment of FIA peaks. Concentrations below the preset limiting value are recorded as negative peaks, whereas those above it are registered as positive peaks.

In addition, use of two identical ISEs inserted in two different streams (one of fixed composition and the other receiving the injected sample) allows the reference electrode to be suppressed and endows the experimental set-up with greater robustness. Finally, a separation unit coupled on-line with the system can be used to boost the selectivity and/or sensitivity and hence expand its scope of application.

Even though the potentiometric sensors (both probe-type sensors housed in a flow-cell, and flow-through sensors proper or tubular electrodes) dealt with in this section do not fit the general features of the sensors discussed in this Chapter (*i.e.* those based on integrated separation and detection), they respond most closely to the underlying principles of ion-sensitive potentiometric sensors (*viz.* an instrumental response to a free energy change associated with mass transfer by ion-exchange, adsorption, liquid-liquid extraction or some other mechanism) than they do to the description of any sensor reviewed in Chapters 3 and 5. The two sections below deal separately with ISEs proper and ISFETs. Their coverage in this book must obviously be quite brief, so only a few representative examples including probe-type and flow-through or tubular sensors are commented on.

4.6.1 Flow-through ion-selective potentiometric sensors

Several classical ion-selective electrodes (some of which are commercially available) have been incorporated into continuous systems via suitable flow-cells. In fact, Lima *et al.* [112] used a tubular homogeneous crystal-membrane (Ag_2S or AgCl) sensor for the determination of sulphide and chloride in natural and waste waters. However, the search for new active materials providing higher selectivity and/or lower detection limits continues. Thus, Smyth *et al.* [113] tested the suitability of a potentiometric sensor based on calix[4]arene compounds for use in flow injection systems. They found two neutral carriers, *viz.* methyl-*p-tert*-butylcalix[4]aryl acetate and

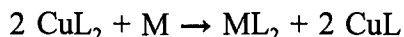
the methyl tetraketone derivative of *p*-*tert*-butylcalix[4]arene to exhibit excellent stability and a rapid response to sodium in a series of carrier streams. They used these membrane electrodes for the determination of sodium in clinical samples and obtained results that compared favourably with those provided by the flame photometry autoanalyser used at a hospital. Recently, trioctyltin chloride incorporated into a PVC membrane was investigated for its potentiometric dynamic response with a view to developing a probe-type sensor for various ions. Some of the limitations imposed by the trioctyltin chloride ionophore were found to be circumvented by using it in an FI system. The membrane was found to provide a Nernstian response and the response time to be quite short; also, sample volumes as low as 30 μL could readily be processed. Some secondary ions delayed baseline restoration after injection, an effect which was not directly related to potentiometric interferences from the ions. However, this adverse effect on analytical performance was overcome by conditioning the electrode with a carrier stream between sample measurements [114]. Several PVC-based liquid membranes containing a quaternary ammonium salt as carrier or a lipophilic macrocyclic pentaamine have also been used to construct selective electrodes for acids and anions housed in wall-jet flow-cells for use in FIA and HPLC [115].

Ishibashi *et al.* devised a potentiometric sensor for the determination of non-ionic surfactants which they improved in several steps. Initially, the authors used a sensor based on a PVC membrane plasticized with 2-nitrophenyl octyl ether that was responsive to cationic complexes formed between a dissolved metal ion and non-ionic surfactants in the sample [116]. At a later stage, they studied the effect of foreign species and elucidated the perturbation from ionic surfactants [117], which they eventually overcame by inserting an ion-exchange column into the base system [118].

Some ISEs containing no inner reference solution, as well as tubular potentiometric sensors, has been used in conjunction with FI systems for the determination of vitamins B₁ and B₆ in pharmaceutical preparations. The membranes used for this purpose were prepared from the vitamin tetra(2-chlorophenyl)borate dissolved in *o*-nitrophenyloctyl ether and immobilized in PVC. The intrinsic behaviour of the tubular electrodes was assessed by using a low-dispersion single-channel FI manifold and compared with those of conventionally-shaped electrodes using the same membrane; the results provided by both were very similar [119].

The use of selective electrodes for indirect determinations of species to which the sensor responds by a reaction involving the analyte as the primary

species is a fairly old practice. Recent approaches include the use of an ISE for the indirect determination of Al(III) and U(IV) in FI manifolds based on a substitution reaction between the determinand and buffered CuL_2 (L = oxalate or malonate ion) at pH 5.4. The potentiometric signal from the sensor is chemically amplified as a result of the stoichiometry of the overall substitution reaction,



which reflects in a doubly Nernstian slope of the Cu(II) electrode function. In this way, aluminium and uranium can be determined over the concentration ranges 1.35–216 $\mu\text{g/mL}$ and 14–2142 $\mu\text{g/mL}$, respectively, with good reproducibility and a high precision but no interference from metal ions forming no complex with the ligand or giving rise to less stable complexes than CuCL_2 . In any case, use of an appropriate masking agent allows Al(III) and U(IV) to be determined in the presence of Fe(III) and Th(IV), respectively [120].

One FI kinetic photometric method using a fluoride ion-selective electrode and a micellar medium allows the determination of phenol drugs (acetaminophen and isoxsuprine) and hydrazino drugs (isoniazid). The chemical foundation of the method is the reaction between 1-fluoro-2,4-dinitrobenzene with the analytes in a weakly alkaline medium, which releases fluoride from the reagent. The slow reactions with phenols were found to be accelerated by the catalytic action of cetyltrimethylammonium bromide (CTAB) micelles. The reaction rate was monitored by using the fluoride ISE housed in a wall-jet cell. The response time and long-term stability of the electrode were found to be adequate for the kinetic determinations undertaken. The linear determination ranges encompassed concentrations between 1×10^{-4} and 50×10^{-4} M; the RSD was 1.8–3.6% and the throughput 20–40 samples/h [121].

The performance of these sensors is markedly influenced by the type of flow-cell in which they are housed. Specialists are aware of this fact, so they usually compare the results provided by cells of various types (wall-jet, thin-layer, tubular) or design their own custom flow-cells in accordance with the characteristics of the system studied. The straightforward high-volume wall-jet cell depicted in Fig. 4.11.A was designed by Lexa and Stulik and tested in unsegmented-flow systems. The cells consists of (1) a cylindrical glass vessel with an opening for (2) the working and (3) the reference electrode, as well as for the jet (4), which are fixed in place by means of polyethylene

O-rings. The horizontal position of the sensor and the jet prevents gas bubbles from adhering to the sensor surface. The glass vessel is furnished with a solution outlet stopcock (5) and placed in the neck of a waste bottle (7): A tube (6) replenishes the solution as needed to maintain a constant level. During sample measurements, the working electrode is shielded from the regenerating solution by the flowing sample solution; air is pumped

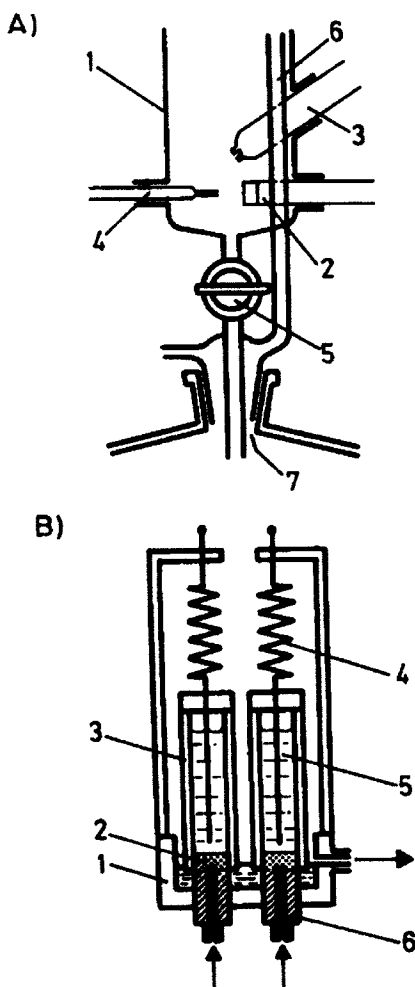


Figure 4.11 — (A) High-volume wall-jet cell. (B) Bubble-through flow-cell. (1) Cell body; (2) membrane; (3) ion-selective electrode; (4) steel spring; (5) inner reference solution; (6) sample guide.

through the jet to agitate the regenerating solution at the surface electrode between samples. The cell performance was assessed by using ordinary fluoride and chloride ISEs and the ensuing method was applied to the determination of both halides in steel corrosion products and a reference sample of fly dust from electric-arc furnaces. The throughput thus achieved was *ca.* 90 measurements/h [122].

Flow-cells accommodating ion-selective electrodes have also been used for continuous-flow measurements in air-segmented streams without debubbling. One such cell is depicted in Fig. 4.11.B. The body was machined from a Plexiglas cylinder (2-cm long \times 5 cm ID). Two Teflon sample guides were screwed into the bottom of the cell body, each having a narrow inlet channel. The indicator and reference electrodes were supported in a vertical position by steel springs serving as connecting wires for the mV/pH meter. The electrodes were positioned in such a way that the flow was introduced at the centre of the membrane face and forced to exit between this and the top surface of the sample guide around the membrane perimeter into the reservoir. Air bubbles passing quickly between the membrane face and the surface of the sample guide never collected under the membrane covering the whole sensing surface, so contact between indicator and reference electrodes was never broken. By using a flow-rate of 4 mL/min, throughputs of 450 samples/h were achieved in chloride and fluoride determinations, respectively, with RSD values better than 2% [123].

Multideterminations with ISEs entail using as many sensors as species are to be determined. Their placement in the hydrodynamic system used is dictated by both their selectivity and the way in which acquired data are to be subsequently processed.

The potential of ISEs for multi-determinations in continuous systems has been exploited by several authors in the form of various cell designs. Thus, Cardwell *et al.* developed the flow-cell depicted in Fig. 4.12.A for the determination of four ions simultaneously with detection by using solid-contact polymer-membrane ion-selective electrodes. The flow-cell design was intended to ensure minimum dispersion and void volumes, a high flow velocity, good contact of the bulk sample plug with each membrane, a well-defined, stable flow across all membranes, and efficient use of the sample plug by making the membranes large relative to the total cell area and arranging them serially in the cell. They cast PVC membranes from tetrahydrofuran solutions onto silver wire contacts in one half of the cell. The membrane compositions were recommended formulations, *viz.* valino-

mycin for potassium, neutral carrier ETH 1001 for calcium and tetradodecylammonium nitrate for NO_3^- ; on other hand, chloride ion was measured by means of an Ag/AgCl electrode. A FORTH program was used to expedite acquisition of the data required for flow injection measurements. The system utility was demonstrated by applying it to the simultaneous determination

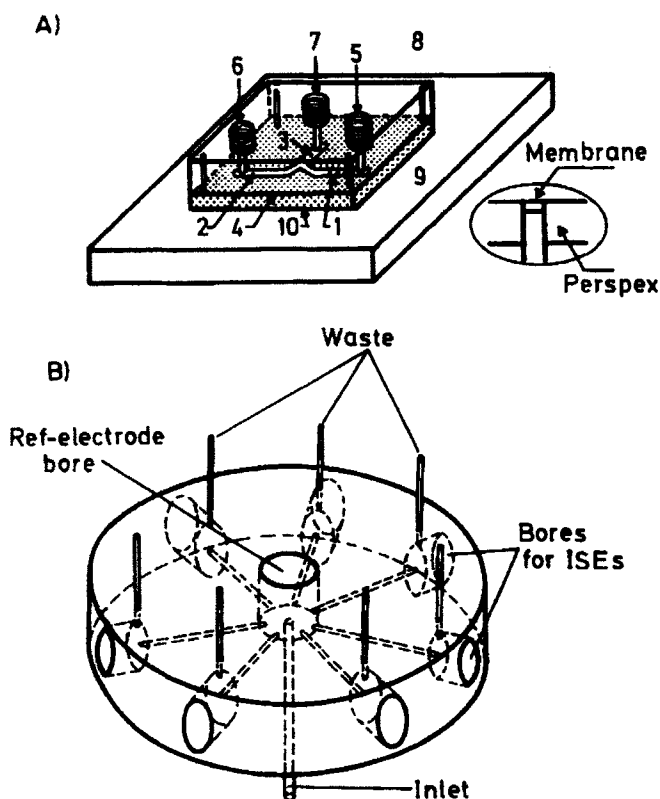


Figure 4.12 — (A) Flow-cell for the simultaneous determination of four analytes: (1) ion-selective electrodes; (2) reference electrode; (3) Pt wire for grounding; (4) Teflon gasket; (5) carrier; (6) inlet for reference solution; (7) waste; (8) screws; (9) diecast box; (10) rubber sheet for sealing. (B) Seven-electrode holder (the reference electrode is placed from the top into the central bore). ISEs for Na^+ , K^+ , Ca^{2+} , NO_3^- , Cl^- , and HCO_3^- (NH_3 electrode with internal buffer of 0.1 mmol/L NaHCO_3) are placed horizontally around the reference electrode, the metal waste tubes being connected to the waste via filter-paper strips. (Reproduced from [124] and [108] with permission of Elsevier Science Publishers and VCH Publishers, respectively).

of potassium, calcium, nitrate and chloride in soil extracts; the results compared well with those afforded by standard procedures (see Table 4.2) [124]. The system was combined with two spectrophotometric transducing flow-cells for the simultaneous determination of potassium, calcium, ammonium, chloride, nitrate and phosphate in a number of plant nutrient solutions [125].

A multi-ion drinking water determination of the principal species analysed for in this type of sample (*viz.* sodium, potassium, calcium, bicarbonate, nitrate and chloride) was developed by taking advantage of commercially available ISEs for all these ions. Figure 4.12.B shows the 7-electrode holder used, which was included in an FI configuration. Because the carrier solution contained 0.1 mM NaHCO_3 , KNO_3 , and CaCl_2 , negative signals were obtained at low concentrations of the corresponding ions. The expeditiousness of the determination (6 ions/min), the small amount of sample needed (200 μL) and the low cost of this assembly defy competition. It is extremely useful for routine screening analysis in this field. Since ISEs have a tendency to detect more in the presence of high concentrations of interfering ions, the screening analysis has a sort of built-in safety device. Samples above a certain limit can thus be put aside for analysis by a more elaborate method [108]

Virtanen constructed an assembly consisting of serially arranged ISEs for the determination of sodium, potassium, calcium and chloride (Fig. 4.13.A) in which the flowing stream impinged laterally on both the working electrodes and the reference electrode placed next to them. The system performance was tested by using solutions simulating the electrolytic composition of serum and regression equations were derived to correct the interference of the other species in the determination of each [126]. A recently reported four-electrode arrangement allows the determination of the four halides. The sensors from chloride, bromide and iodide ion were constructed from compressed pellets of $\text{Ag}_2\text{S}/\text{AgX}$ ($\text{X} = \text{Cl}^-$, Br^- or I^-) that were drilled lengthwise (1.5-mm holes) to make them suitable for use in an FI manifold. A column of AgCl was included in the manifold used for chloride (Fig. 3.13.B) in order to avoid the interferences from bromide and sulphide ions. Also, a column of amalgamated lead was used in the manifold for bromide to overcome the interfering effects of iodide, sulphide and chloride. Neither column nor addition of ascorbic acid was required for the determination of iodide. These three sensors plus a commercially available

Table 4.2. Results obtained in the determination of four ions in soils by using the sensor of Cardwell *et al.* and various standard methods

Sample number	Concentration found ^(a) (mg/L)							
	Chloride		Nitrate		Potassium		Calcium	
	IC	FIA	IC	FIA	AAS	FIA	AAS	FIA
1	57.6	57.9 (3.2)	177	154 (0.8)	98.3	94.9 (1.3)	56.4	59.2 (0.8)
2	28.8	30.6 (2.4)	254	225 (1.3)	61.0	52.7 (1.0)	61.5	65.0 (2.3)
3	22.2	19.3 (1.8)	144	125 (1.3)	50.1	41.7 (0.3)	40.5	44.1 (1.9)
4	45.9	57.9 (1.8)	911	865 (0.7)	273	278 (0.4)	181	183 (1.0)
5	47.1	47.2 (1.3)	399	388 (1.2)	238	237 (0.8)	114	110 (1.2)
6	101	97.2 (2.3)	644	617 (0.8)	241	244 (0.3)	218	217 (1.0)
7	20.4	20.3 (2.9)	243	213 (0.9)	209	203 (0.3)	173	154 (1.2)

^(a) Values in brackets are the relative standard deviations ($100\sigma/\text{mean}$) for 4–5 determinations
(Reproduced from [124] with permission of Elsevier Science Publishers)

fluoride sensor were used in a mixed serial-parallel manifold for the simultaneous determination of the four analytes [127].

The selectivity of ISEs used in flow systems can be boosted by incorporating an on-line separation unit (e.g. an ion-exchange column, a dialysis module or a gas-diffusion device) into the hydrodynamic system. Occasionally —particularly when an ion-exchange column is used—, the ancillary

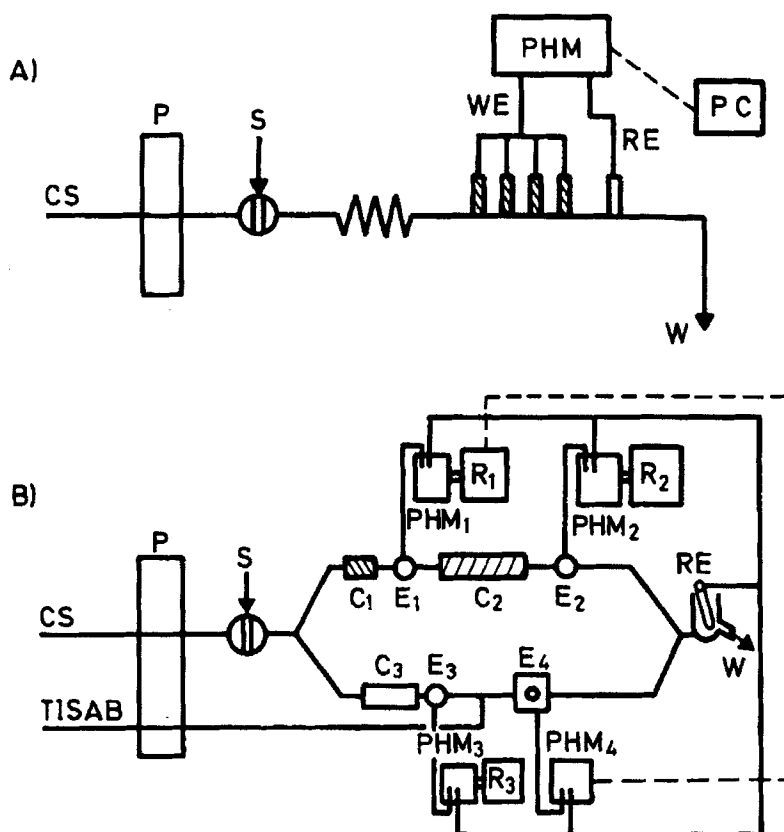


Figure 4.13 — (A) Serially arranged ISEs for the simultaneous determination of sodium, potassium, calcium and chloride ions. (B) Serially and parallelly arranged ISEs for the simultaneous determination of I^- , Br^- , Cl^- and F^- . WE working electrode; PC personal computer; E_1 – E_4 I^- , Cl^- , Br^- and F^- ISEs, respectively; C_1 , C_3 amalgamated-lead columns (1.5 and 2.5 cm long, respectively); C_2 AgCl column; RE reference electrode; PHM pH/mV-meter; S sample injection; CS carrier stream; P pump; R chart recorder; W waste. (Reproduced from [126] and [127] with permission of Elsevier Science Publishers and Pergamon Press, respectively).

unit is employed with a view to improving the sensitivity (through preconcentration) rather than the selectivity.

Risinger incorporated *ion-exchange* into the manifold of Fig. 4.14.A in order to improve the determination of copper by using an ISE for the metal ion and a column containing 8-quinolinol immobilized on porous glass for

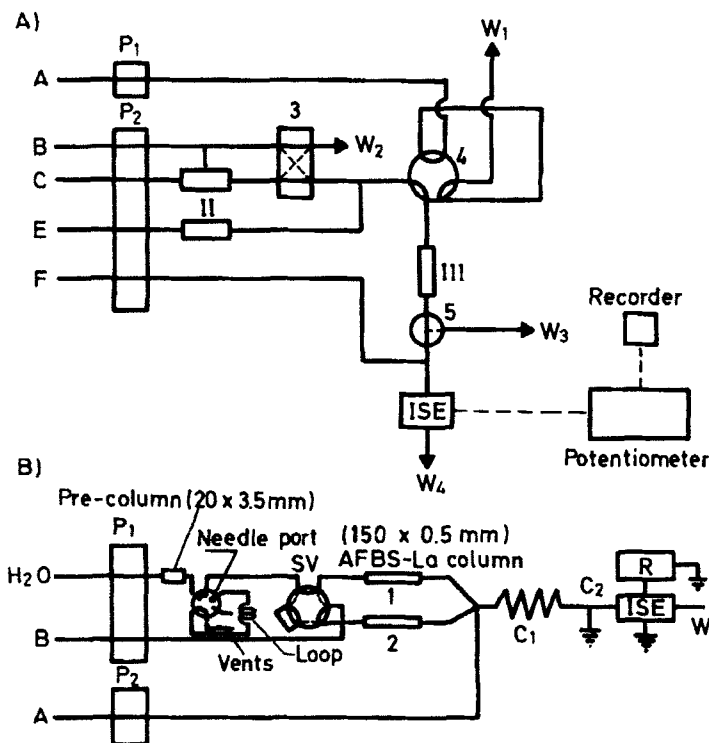


Figure 4.14 — (A) Flow injection system for the preconcentration and determination of copper: P₁, P₂, peristaltic pumps; A 0.5 M HNO₃; B sample ($q = 2.5$ mL/min); C water ($q = 0.5$ mL/min); E 1 M NaNO₃/0.1 M NaAcO, pH 5.4 ($q = 0.5$ mL/min); F 1 M NaAcO/ 2×10^{-7} M Cu²⁺, pH 5.0 ($q = 1.0$ mL/min); 3–5 valves; ISE copper ion-selective electrode; W waste; I and II 2 and 3 mL of chelating ion exchanger for purification; III 100 μ l of chelating ion exchanger for metal ion preconcentration. (B) Scheme of the flow system for the determination of halides: A 4 M HAcO/1 M NaCl/0.57 ppm F⁻; B 1 M NaOH/0.5 M NaCl; C₁ mixing coil (1 m \times 0.5 mm ID PTFE tube); C₂ stainless-steel tube (5 cm \times 0.5 mm ID); ISE ion-selective electrode; R recorder. (Reproduced from [128] and [129] with permission of Elsevier Science Publishers and the Royal Society of Chemistry, respectively).

preconcentration of the analyte. Two additional columns packed with commercially available chelating ion-exchangers (I and II in Fig. 4.14.A) were used for on-line removal of trace metals from the carrier and buffer. Samples were inserted into the buffer stream by means of a time-based injection device and the metal ions bound to the immobilized ligand while anions and inert sample components passed onto waste without contacting the electrode. A volume of acid was then injected to elute the analyte into a neutralizing buffer circulated through the electrode. The detection limit thus achieved was 10^{-7} M for 5 mL and 3×10^{-8} M for 25 mL of sample, and the corresponding maximum throughputs were 12 and 5 samples/h, respectively [128].

Okabayasi *et al.* used two identical columns containing Alizarin–Fluorine Blue sulphonate–lanthanum complex in an alternate fashion in order to increase the sample throughput in the preconcentration/separation of fluoride prior to its determination by use of an ISE (Fig. 4.14.B). By switching valve SV, the analyte in a sample was measured and eluted while that in the next sample was retained in the other column [129].

In the method proposed by van Staden for the determination of three halides, these are separated in a short column packed with a strongly basic ion-exchange resin (Dowex i-X8) that is placed in an FI manifold. A laboratory-made tubular silver/silver halide ion-selective electrode is used as a potentiometric sensor. Van Staden compared the response capabilities of the halide-selective electrodes to a wide concentration range (20–5000 $\mu\text{g/mL}$) of individual and mixed halide solutions in the presence and absence of the ion-exchange column. By careful selection of appropriate concentrations of the potassium nitrate carrier/eluent stream to satisfy the requirements of both the ion-exchange column and the halide-selective electrode, he succeeded in separating and determining chloride, bromide and iodide in mixed halide solutions with a detection limit of 5 $\mu\text{g/mL}$ [130].

Dialysis units provided highly efficient means for increasing selectivity in a dynamic system by placement in front of a lithium-selective electrode constructed by incorporating 14-crown-4 ether 3-dodecyl-3-methyl-1,5,8,12-tetraoxacyclotetradecane into a PVC membrane that was in turn positioned in a microconduit circuit by deposition on platinum, silver or copper wires. The circuit was used to analyse undiluted blood serum samples by flow injection analysis with the aid of an on-line coupled dialysis membrane. For this purpose, a volume of 200 μL of sample was injected into a de-ionized water carrier (donor) stream and a 7 mM tetraborate buffer of pH 9.2 was

used as the acceptor stream. The lithium-to-sodium transfer ratio across the membrane was 0.57, and the overall selectivity for lithium relative to sodium was 45–49 when using the matched-potential method with silver or copper wire electrodes. Serum samples analysed with the FI/electrode assembly using the copper wire electrode provided results similar to those obtained by atomic absorption spectrometry, with an average error of -3.1% for pooled serum standards and -6.6% for aqueous standards [131].

Van Staden reported a rapid, reliable automated method for direct measurement of the chloride content in milk based on the principles of flow injection analysis and the use of a dialyser to remove interferents. Dialysed chloride was measured by means of a coated tubular chloride ion-selective electrode. Potential changes arising from the interference of casein were thus avoided and baseline stability ensured. The results obtained for chloride in milk compared well with those provided by standard recommended methods. The linear range for chloride was 250–5000 $\mu\text{g/mL}$ for 30 μL of sample, and the coefficient of variation was better than 0.5%. The throughput was *ca.* 120 samples/h [132].

Separations of gaseous species (and others than can readily be converted into gases) by *diffusion* are highly selective since normal samples typically contain few such components [133].

As regards ammonia, groundwater treatment plants regularly require a knowledge of the ammonium ion content in the incoming water; this is a variable parameter, particularly in densely populated, highly industrialized hydrographic basins. It is also one of the parameters that must be measured in order to determine the chlorine dose to be added in the treatment process. A method allowing the ammonium ion concentration in the incoming waters to be monitored in a continuous fashion can be used to determine the exact amount of chlorinating agent to be added, thereby saving resources and improving water quality. This is the source of the wide use of ammonium ISEs in continuous systems for this purpose. A specially constructed all-solid state tubular flow-through ammonium electrode was used by Alegret *et al.* in conjunction with a gas-diffusion chamber to enhance its selectivity. The electrode, a modified design of other flow-through tubular PVC matrix membrane electrodes containing no inner reference solution [134–136], is shown in Fig. 4.15.A. The conductive support onto which the membrane was applied was graphite-loaded epoxy resin. The inner diameter of the channel drilled in the conducting support was 1.5 mm and once the sensing membrane layers had been deposited dropwise, the inner diameter of the

tubular channel was reduced to *ca.* 1.2 mm (membrane thickness *ca.* 0.15 mm). The FI manifold into which the sensor was incorporated is depicted in Fig. 4.15.B. The sample was inserted into a distilled water stream that was merged with a basic stream in order to ensure the presence of ammonia as such on passage through the gas-diffusion cell. The acceptor stream consisted of Tris buffer that drove the analyte to the detector. Up to 30 samples/h were processed with a detection limit of *ca.* 10^{-6} M. The system was tested with

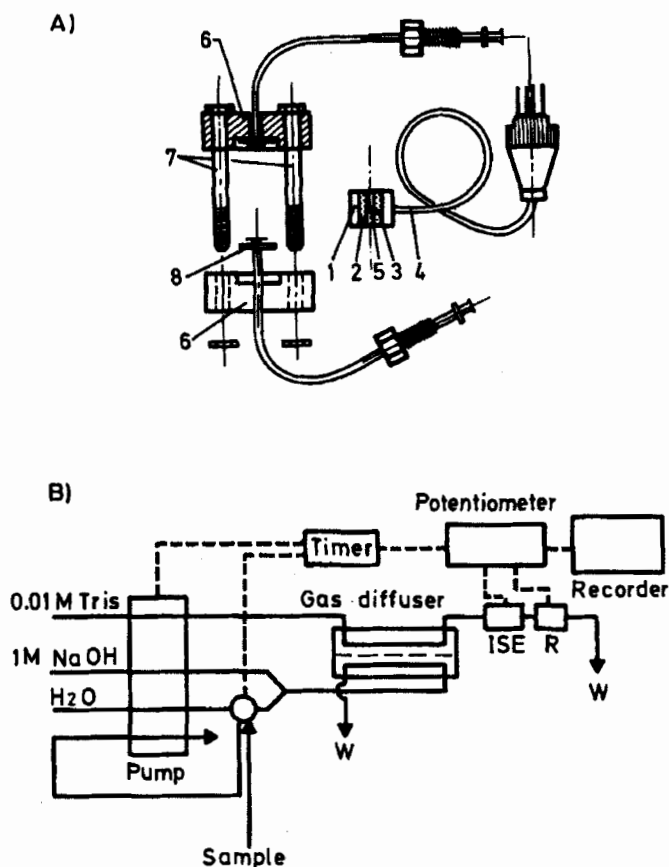


Figure 4.15 — (A) Tubular flow-through electrode: 1 Perspex body; 2 conducting epoxy cylinder; 3 mobile carrier PVC membrane; 4 electric cable; 5 channel (1.2 mm ID); 6 holders; 7 screws; 8 O-rings. (B) Schematic diagram of a system for on-line monitoring of ammonia: ISE tubular flow-through ammonium ion-selective electrode; R reference electrode; W waste. (Reproduced from [137] with permission of the Royal Society of Chemistry).

excellent results on a water treatment plant [137]. A similar configuration including a tubular AgI/Ag₂S electrode was employed for the determination of free and weakly complexed cyanide [138].

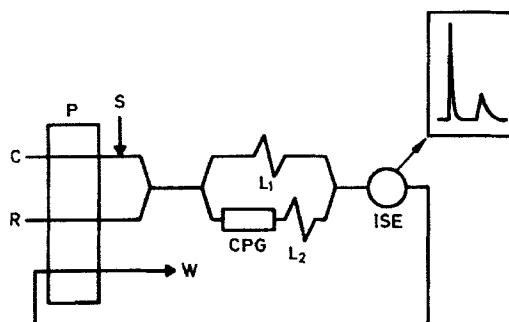


Figure 4.16 — Schematic diagram of a split-stream FI system used for the determination of glutamine in bioreactor media: C de-ionized water carrier stream; R buffer diluent reagent stream; S sample injection point; L₁, L₂ delay coils; CPG controlled pore glass enzyme reactor; ISE ammonium ion-selective membrane electrode; W waste. (Reproduced from [139] with permission of the American Chemical Society).

Application of this type of electrode has been extended to analytes capable of yielding the monitored species, as in the determination of L-glutamine in bioreactor media by using a continuous configuration where the sample injection unit is followed by an enzyme reactor (glutaminase immobilized on CPG) that catalyses conversion into ammonia. Errors arising from the presence of endogenous ionic interferents can be avoided by using a configuration such as that shown in Fig. 4.16 in order to use a high background level of interfering ions in the sample diluent/carrier stream to convert the normally logarithmic potentiometric sensor into a linear detector over a given concentration range of primary ions. A split-stream single-detector assembly provides a convenient means for offsetting varying levels of background interfering ions in the injected samples. One portion of the split stream passes directly to the ion-electrode detector and gives a signal that is linearly related to the concentration of endogenous primary ions in the sample. The second portion of the split sample is delayed while passing through an immobilized enzyme bed that generates electrode-detectable primary ions in proportion to the concentration of the substrate analyte in

the sample. Two linear equations in two unknowns describe the twin potentiometric responses observed. The underlying concept was demonstrated by the accurate determination of L-glutamine in hybridoma bioreactor media using an ammonium ISE that was constructed by incorporating nonactin into a plasticized PVC membrane [139].

Miniaturization trends have also reached ISEs, as shown by Hongbo's atropinium and scopolaminum microelectrodes integrated in a microconduit system. Figure 4.17.A depicts the tubular flow-cell used. Both microelectrodes were constructed by replacing part of the inner walls of a small piece of PVC tubing (1.5 cm long, 0.5 mm ID, 1.5 mm OD) with the conforming sensing membrane (0.25 mm²). They were placed into a Perspex housing as shown in Fig. 4.17.A and the housing was filled with 0.1 M NaCl plus 10⁻² M atropinium or scopolaminum inner solution containing saturated AgCl, into which an Ag/AgCl wire electrode was inserted to serve as the inner reference system. The cell was incorporated into the integrated microconduit circuit shown in Fig. 4.17.B. The whole continuous assembly is depicted in Fig. 4.17.C. This miniaturized system allows the determination of both active principles in drugs at near-equilibrium and a rate of 120 samples/h [140].

4.6.2 Sensors based on ion-selective field-effect transistors

Ion-selective field-effect transistors (ISFETs) are the offspring from the marriage of ion-selective electrodes and solid-state electronics. They avoid the noise problems of ISEs arising from the high electric impedance of the ion-selective membrane used on bringing it into contact with the amplifier. There are two types of ISFETs: metal-insulator capacitors and transistors. The semiconductors used are normally silicon and the insulators silicon dioxide. These semiconductor structures are called MOS devices. An iridium-metal oxide semiconductor capacitor was described above in dealing with sensors integrating gas-diffusion and detection [16–20], so only a single ISFET design employed in flow systems is discussed here.

Ever since an ISFET that was chemically modified by a valinomycin-containing PVC membrane was reported [141], there has been general consensus on the advantages of this type of microsensor over conventional ISEs. Some serious problems have also been acknowledged, though (*e.g.* the low mechanical stability of the membranes, the interference of CO₂ in the potentiometric response, the lack of a stable micro-reference electrode and the relatively high drift rate of ISFETs). Attachment of the membrane can

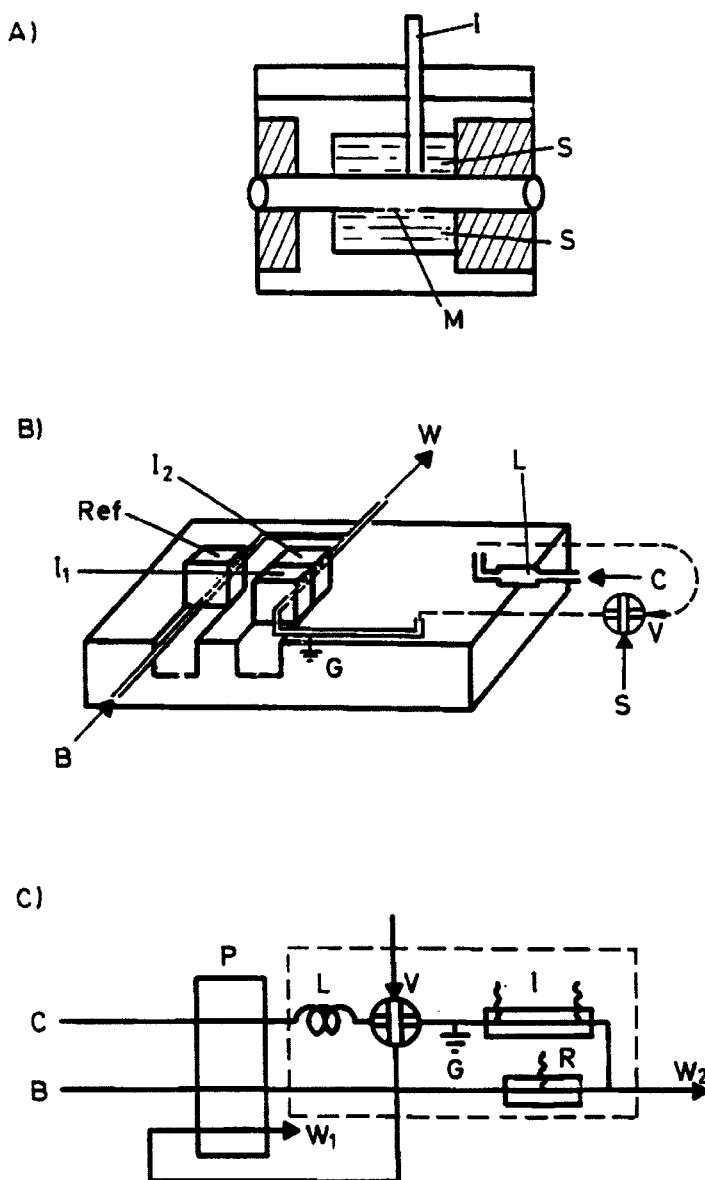


Figure 4.17 — (A) Exploded view of a tubular flow-cell integrated microconduit system. I Ag/AgCl inner reference electrode; M sensitive membrane; S internal reference solution. (B) Detail of the integrated microconduit shown within the dotted lines in C. (C) Integrated-microconduit FI manifold for potentiometric measurements: C carrier stream; R reference electrode solution; P pump; V injection valve; I indicator electrode; R reference electrode; I pulse inhibitor; G ground; W waste. (Reproduced from [140] with permission of Pergamon Press).

be improved by mechanical [142] or chemical anchoring [143,144] to the surface of the oxide gate. Also, the interfering effect of CO₂ on the analytical response can be overcome by using an intermediate buffered hydrogel layer [145,146]. On the other hand, the lack of a stable micro-reference electrode can be circumvented by combining a reference field-effect transistor (FET) and one or two chemically modified FETs (CHEMFETs) for differential measurements relative to a *pseudo*-reference electrode [147–149]. Finally, the drift rate can be lowered by incorporating ISFETs into FI systems, which are highly suitable for automatic continuous monitoring and enable discrete sequential analyses in near-real time. The surface of a sensor inserted in an FI manifold is continuously cleaned by the carrier solution, so the baseline is smoothly restored after each sample injection as a result of the drift being neglected in computing the analytical signal. The enhanced performance provided by flow systems has fostered the development of customized cells and comparative assessment of their effectiveness.

One of these cells (of the wall-jet type) was used to make differential measurements in an FIA configuration such as that shown in Fig. 4.18.A. The cell (Fig. 4.18.B) consisted of polycarbonate blocks with the ISFETs (3 and 4 in the Figure) inserted in such a way that the gate covered with the ion-sensitive material lay accurately behind a conically shaped hole (45° angle) having a smallest diameter of 1 mm. Spring contacts bridged the copper leads to the wiring from the ISFET amplifier. The flowing stream entered a small chamber opposite the gate surface via a nozzle of 0.15 mm ID. The distance between the nozzle and the CHEMFET surface was 6 mm. Three outlets were used separately to facilitate filling of the cell and prevent build-up of air bubbles. The output signal from the two CHEMFETs was evaluated in a differential measurement set-up using a platinum tube as a *pseudo*-reference electrode (5 in Fig. 4.18.B). The optimized FI system exhibited a linear response of 56 mV per decade for potassium concentrations above 5×10^{-5} M and provided favourable results in the determination of the element in human serum and urine [150].

Cobben *et al.* [151] designed and tested a wall-jet and a flow-through cell of this type. The wall-jet cell (Fig. 4.19.A) consisted of two parts, A and B. Part A was a Perspex block of $24 \times 24 \times 20$ mm³ (1) furnished with two resilient hooks (3) for electrical contact. The hooks were pressed on the surface of the contact pads of the CHEMFET (4), the back of which lay on the Perspex surface. In this way, the sensor gate was positioned in the centre of the Perspex block, which was marked by an engraved cross. Part B was

also a Perspex block (2) of the same dimensions into which a glass tube (5) was mounted. The glass tube (1.6 mm ID, 8 mm OD) was fitted with a ring (6) held by the block. The front of the tube was modified in two ways. At the top, a cut-away was made to provide enough space for the hooks that stood out of the Perspex block. At the bottom, a hole of 3 mm ID leading to waste was made. A PTFE tube (70 mm long, 0.3 mm ID, 1.6 mm OD) (7) was drawn inside the glass tube to form a nozzle at a distance of 3 mm from the sensor surface. The two blocks were held together by four pins with springs and nuts.

The flow-through cell developed by Cobben *et al.* (Fig. 4.19.B) also consisted of two Perspex blocks, A and B. Part A was identical with that used in the previous wall-jet cell, whereas part B (2) was a knotted block ($24 \times 24 \times 15 \text{ mm}^3$) into which two holes were drilled towards the centre

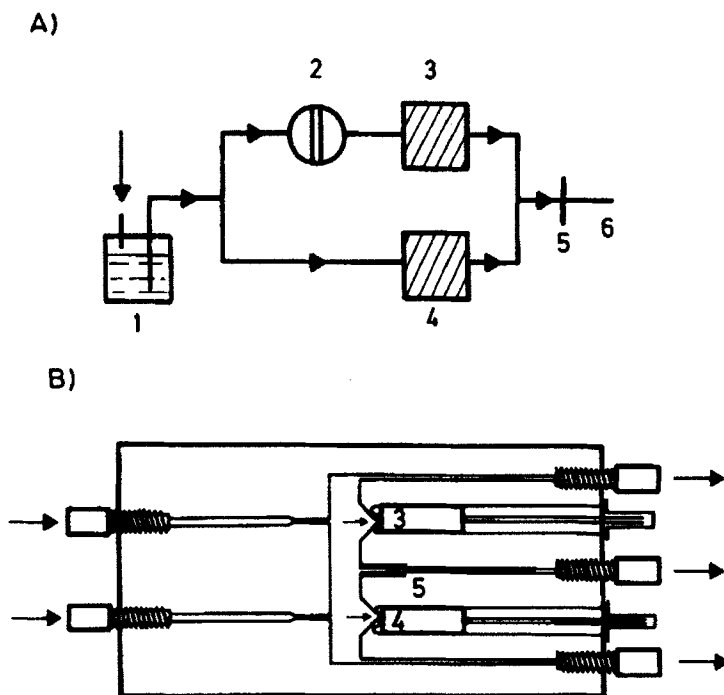


Figure 4.18 — (A) Schematic diagram of a flow-injection system for potassium: (1) carrier solution; (2) injection valve; (3,4) flow cell; (5) *pseudo*-reference electrode; (6) waste. (B) Detail of the flow-cell: (3,4) CHEMFETs; (5) *pseudo*-reference electrode. (Reproduced from [150] with permission of Elsevier Science Publishers).

of the bottom. The cell thus obtained was V-shaped. The diameter of the flow channels was 0.5 mm and match that sensor gate width. Both this and the wall-jet cell were used in a manifold such as that depicted in Fig. 4.18.A, where the carrier was propelled to the two flow-cells (3 and 4) by nitrogen pressure. The flow-cells used were both of the wall-jet or the flow-through type and furnished with a potassium ion-sensitive CHEMFET. The cell contents were measured differentially with respect to a platinum *pseudo*-reference electrode(5) inserted in the combined waste stream. A comparison of the performance of the two cells revealed that the peaks obtained with the wall-jet cell were less symmetric than those provided by the flow-through cell.

Figure 4.20.A shows a more recent cell reported by Cobben *et al.* It consists of three Perspex blocks, of which two (A) are identical and the third (B) different. Part A is a Perspex block (1) furnished with two pairs of resilient hooks (3) for electrical contact. With the aid of a spring, the hooks press at the surface of the sensor contact pads (4), the back side of which rests on the Perspex surface, so the sensor gate is positioned in the centre of the block, which is marked by an engraved cross as in the above-described wall-jet cell. Part B is a prismatic Perspex block (2) ($85 \times 24 \times 10 \text{ mm}^3$) into which a Z-shaped flow channel of 0.5 mm diameter is drilled. Each of the wedges of the Z reaches the outside of the block. The Z-shaped flow-cell thus built has a zero dead volume. As a result, the solution volume held between the two CHEMFETs is very small ($3 \mu\text{L}$). The cell is sealed by gently pushing block A to B with a lever. The inherent plasticity of the PVC membrane ensures water-tight closure of the cell. The closeness between the two electrodes enables differential measurements with no interference from the liquid junction potential. The differential signal provided by a potassium-selective and a sodium-selective CHEMFET exhibits a Nernstian behaviour and is selective towards potassium in the presence of a (fixed) excess concentration of sodium. The combined use of a highly lead-selective CHEMFET and a potassium-selective CHEMFET in this type of cell also provides excellent results.

Alegret *et al.* devised a pH ISFET based on a flow-through cell designed by themselves and an FI manifold including a gas-diffusion module for the on-line separation of gaseous analytes with acid-base properties. In this way, they obtained a linear determination range of 1×10^{-4} – 1×10^{-2} M for ammonia and 7×10^{-5} – 4×10^{-3} M for sulphur dioxide, with an RSD of 1% and 0.5%, respectively [153].

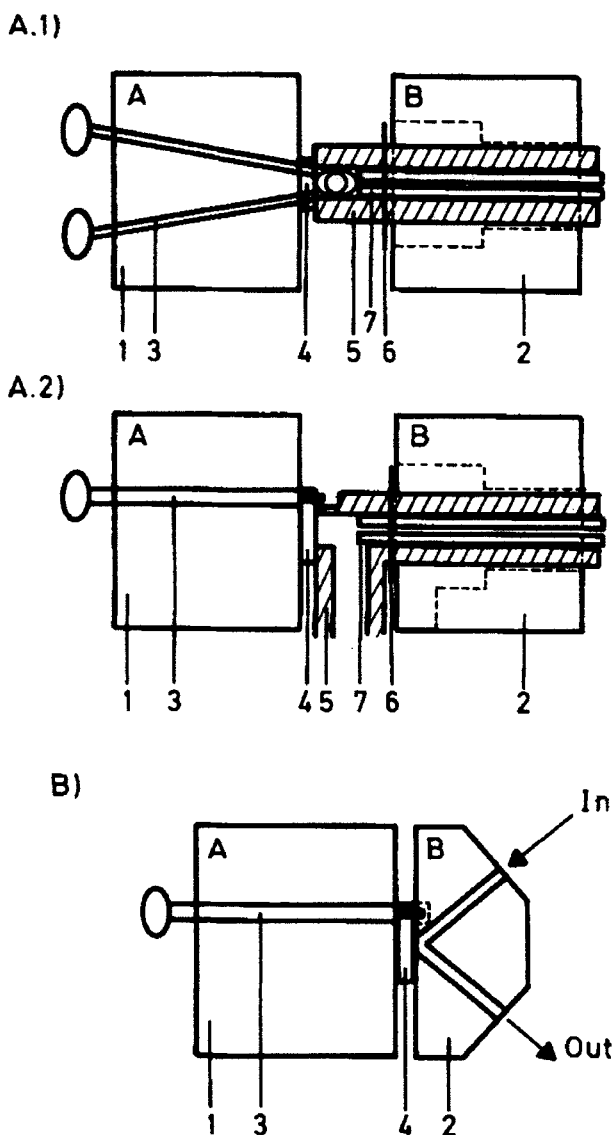


Figure 4.19 — (A) Detail of a wall-jet cell in (A1) top view and (A2) side view: (1,2) Perspex blocks; (3) contact wire (hook); (4) CHEMFET; (5) glass; (6) ring; (7) PTFE tubing. (B) Side view of the flow-through cell: (1,2) Perspex blocks; (3) contact wire; (4) CHEMFET. (Reproduced from [151] with permission of Elsevier Science Publishers).

The ISFET-based integrated coulometric sensor-actuator system was introduced in 1985 [154] in order to facilitate *in situ* calibration of ISFETs. The essential components of a prototype sensor based on this operational principle are shown in Fig. 4.20.B. The system was built by integrating a large noble-metal actuator electrode and a counter-electrode in a piece of silicon. A window in the actuator electrode was etched to receive the gate of the ISFET, which functioned as a pH indicator. The flow-through cell was constructed by sealing a silicon cover with an etched cavity of the chip. The system operation resembles that of a conventional coulometric titration system very closely. The sample was first injected into the cavity and the

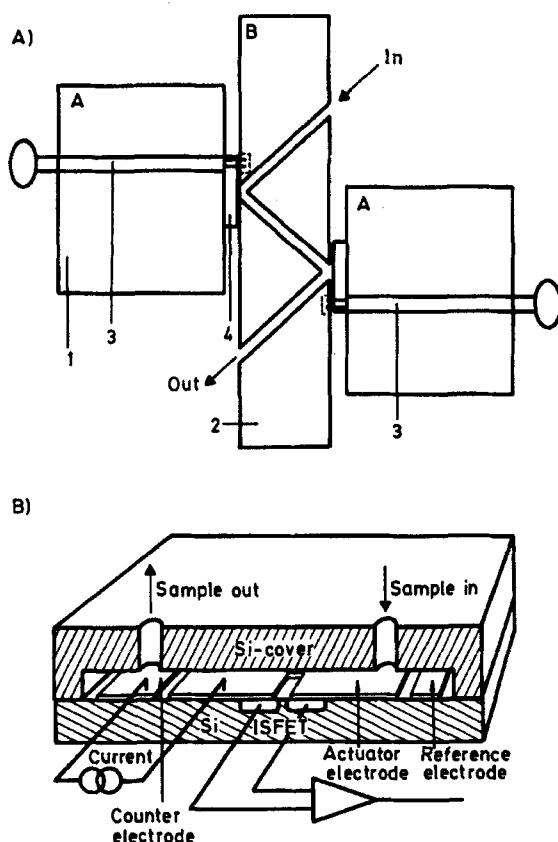


Figure 4.20 — (A) Detailed side view of a double-sensor flow-cell: 1,2 Perspex; 3 contact wire; 4, CHEMFET. (B) Scheme of a microlitre coulometric sensor. (Reproduced from [152] and [154] with permission of Elsevier Science Publishers).

current was then applied to the actuator electrode and counter-electrode. By using coulometrically generated protons or hydroxyl ions, acid and base titrations can be performed highly accurately. The amount of electric charge used for a coulometric titration is roughly proportional to the sample concentration since the titration involves virtually the whole bulk solution. The main advantage of this system arises from the fast response of the ISFET used as pH sensor in the coulometric titration. In addition, the system is highly miniaturized thanks to the silicon technology used and the sample volume required is substantially reduced as a result. Related applications include construction of a low-drift carbon dioxide sensor [156,157] and the development of theoretical treatments for description of these systems [158–160].

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5

Flow-through sensors based on integrated reaction, separation and detection

5.1 INTRODUCTION

The previous two chapters describe flow-through sensors where detection takes place simultaneously with the derivatizing analytical reaction (Chapter 3) and the separation–elution of the analytes or their reaction products (Chapter 4). This Chapter deals with sensors where the three key processes involved (reaction, separation and detection) occur in the same sensitive microzone, which is located in a special flow-cell that is coupled on-line to a continuous-flow configuration [1] intended to: (a) transport the samples and/or reagents; (b) condition the sample in order to ensure optimal conditions for sensing; (c) regenerate the sensor (if it is of the irreversible–reusable type) between successively analysed samples; and (d) facilitate preservation of the sensor while not in use. These continuous-flow configurations are usually similar to those depicted in Figs 2.13 and 2.14, and rely on the fundamental principles of flow injection analysis [2].

Most of the sensors discussed in this Chapter involve immobilization of a reagent and/or catalyst at the active microzone; occasionally, however, no active ingredient of the (bio)chemical reaction is immobilized (*e.g.* when the reaction takes place in the solution held in the sensor flow-cell). The immobilized reagent can play a single or twofold role by acting as an ingredient of the derivatizing reaction and/or facilitating separation.

These sensors can be classified according to various criteria. One classification is based on space and time concepts in relation to the processes taking place at the sensing microzone (*viz.* whether they occur sequentially or simultaneously). As can be seen in Fig. 5.1, (a) mass transfer (of analytes

or interferents) may take place prior to the analytical reaction, which is simultaneous with detection; (b) a (bio)chemical reaction may be introduced prior to simultaneous separation and detection; and (c) all three processes may be simultaneous, so no space or time discrimination between them will be possible.

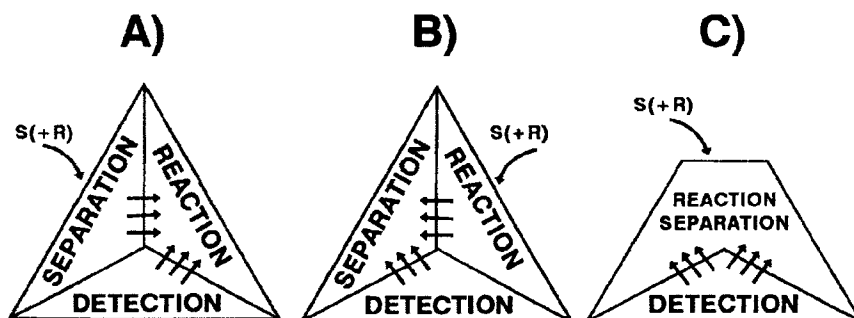


Figure 5.1 — Classification of (bio)chemical flow-through sensors based on integrated reaction, separation and detection according to whether the three processes take place sequentially (A,B) or simultaneously (C) at the sensing microzone. S sample; R reagent. (Reproduced from [1] with permission of the Royal Society of Chemistry).

One other classification is based on the type of separation involved, which dictates the sensing microzone design to be used. Mass transfer can be effected across a membrane separating two liquids, one allowing passage of gases (gas diffusion) or ions/molecules (dialysis), or in a chemically active solid support (sorption). The species to be transferred between phases may be any of the ingredients of the analytical reaction, *viz.* the analyte, reagent, catalyst or product. Figure 5.2 shows these three alternatives in graphical form. This classification is used throughout this Chapter in discussing the most salient achievements in this context.

The reversibility of the sensing process is determined by the features of the (bio)chemical reaction, immobilized species (reagent and/or catalyst) and separation process involved. Very often, the slow kinetics of some such processes make them apparently irreversible in practice when in fact they are not in theory. Many of these sensors are of the irreversible–reusable type and require two steps (sensing and regeneration) for proper functioning. Only

if the immobilized reagent is depleted after a greater or lesser number of determinations should an irreversible sensor be used in this context. As a result, no disposable (single-use) flow-through sensors have so far been developed.

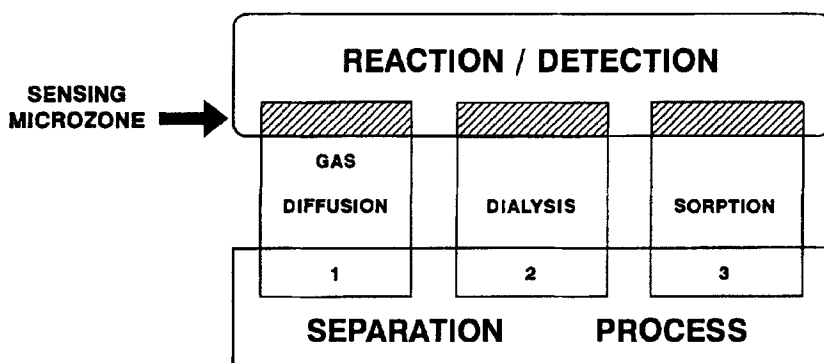


Figure 5.2 — Classification of (bio)chemical flow-through sensors based on integrated reaction, separation and detection according to the type of separation technique involved.

Figure 5.3 shows the different possible ways in which the ingredients of the (bio)chemical reaction can take part in the sensing process. For example, the analyte can be retained temporarily and take part in the separation process. The reagent can be present in the solution used to immerse the sensor or immobilized in a permanent fashion on a suitable support. Also, the catalyst can be introduced directly across a membrane or be permanently immobilized. Finally, the reaction product can be the species transferred in the separation process or also be temporarily immobilized. These and other, more specific alternatives that are described below are all possible in (bio)chemical flow-through sensors integrating reaction, separation and detection.

For a detailed description of the separation processes that may take place at the sensing microzone, the foundation of which is closely related to non-chromatographic continuous separation techniques based on mass transfer across a gas–liquid (gas diffusion), liquid–liquid (dialysis, ultrafiltration) or liquid–solid interface (sorption), interested readers are referred to specialized monographs (*e.g.* [3]).

5.2 INTEGRATION OF GAS-DIFFUSION, REACTION AND DETECTION

The fact that the species transferred across the sensor membrane (the analyte or reaction product) must be a gas limits application of this type of flow-through sensor, which, however, is still more versatile than are the sensors based on integrated separation (gas diffusion) and detection [4] described in Section 4.2; in fact, while these latter can only exploit physico-chemical properties of the analytes transferred, sensors based on triple integration allow the implementation of a (bio)chemical reaction and formation of a reaction product, so they are applicable to a much wider variety of systems with adequate sensitivity and selectivity.

In these sensors (Fig. 5.2.1), separation across a diffusion membrane takes place at the sensing microzone before reaction and detection, both of which occur simultaneously. The species transferred can be the analyte or the gaseous product resulting from it; either can be immobilized temporarily on the sensing microzone containing the reagent. Two reagents are occasionally needed to form the gaseous product and a suitable product for

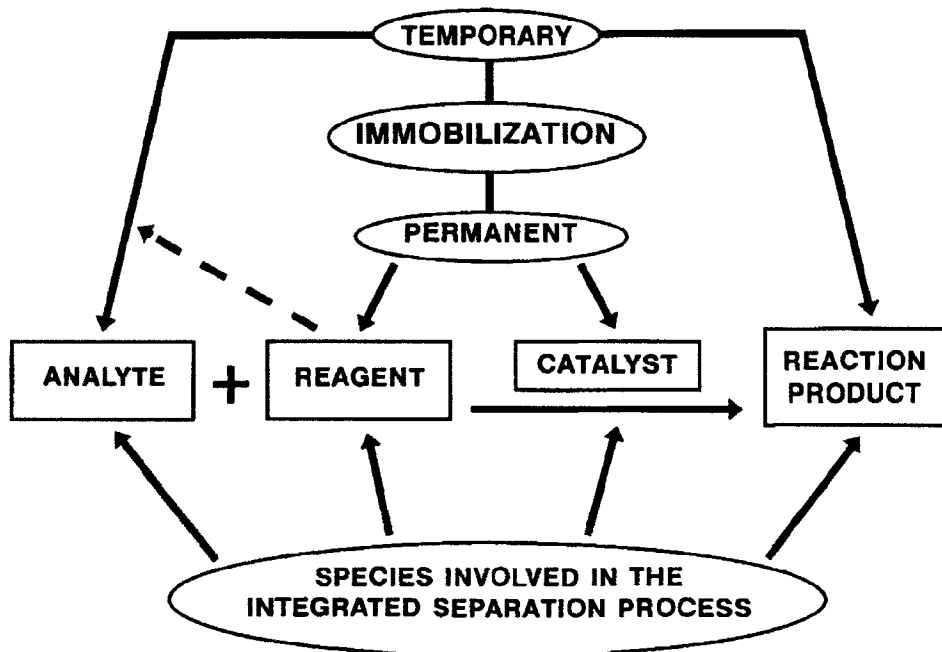


Figure 5.3 — Types of immobilization and species involved in (bio)chemical flow-through sensors integrating reaction, separation and detection.

detection. The derivatizing reagent may be permanently immobilized on an appropriate support or the gas-diffusion membrane itself —alternatively, it can be included in the acceptor stream that is passed over the sensing microzone.

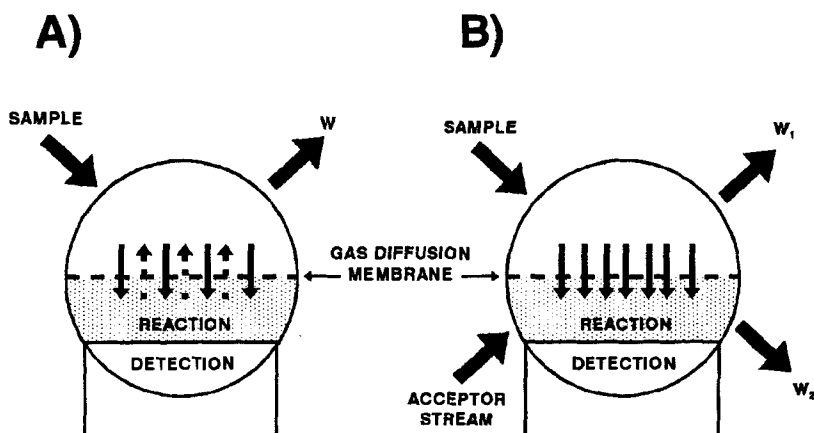


Figure 5.4 — Types of (bio)chemical flow-through sensors involving gas diffusion according to the number of streams that are passed over the sensing microzone. (A) One stream. (B) Two streams. W waste. For details, see text.

As shown in Fig. 2.10, membrane flow-through sensors based on integrated reaction, separation and detection are constructed in two different designs depending on whether the sample (donor) stream is to be circulated alone or in conjunction with the acceptor stream, where reaction and detection are integrated (Fig. 5.4). In the configuration depicted in Fig. 5.4.A, the analyte (or its reaction product) crosses the diffusion membrane in one direction and these two ingredients plus other products may or may not traverse it in the opposite direction since only a sample stream is passed over the sensing microzone. In the miniature sensor of Fig. 5.4.B, similar to an ordinary gas-diffusion cell, reaction and detection take place in an additional (acceptor) stream where the reagent can be included if it is not used in immobilized form. The former sensor has the disadvantage that the sensing microzone may occasionally be saturated with analytes and hence usable for

a limited number of samples and standards only. This shortcoming is normally avoided by the latter sensor as a result of the flushing/renewal action of the acceptor stream used.

The analytes typically determined by using this type of sensor are those usually addressed by gas-diffusion systems, *viz.* ammonia (or ammonium ion), carbon dioxide (or carbonates) and oxygen. The detection system used is most frequently photometric, fluorimetric or potentiometric, and can be integrated with or connected to the sensing microzone. The description below is based on the two choices shown in Fig. 5.4.

5.2.1 Sensors based on a single sample-donor stream

In this type of sensor (Fig. 5.4.A), the sensing microzone accommodating the gas-diffusion membrane is traversed by a stream of (a) a carrier intended to transport the injected sample, condition it (*e.g.* adjusting its pH) and prepare/regenerate the sensor; or (b) the sample itself, introduced into the continuous manifold by aspiration or injection and subsequently mixed in a continuous fashion with another stream if needed.

In 1981, Seitz *et al.* [5] reported a flow-through sensor for the determination of oxygen in gases and water by chemiluminescent reaction with 1,1',3,3'-tetraethyl- Δ -2,2'-di(imidazolidine) (EIA). The sensor consisted of a mini-chamber holding a solution of EIA in *n*-hexane that was magnetically stirred by means of a mini-stirring bar. The chamber was located between a photomultiplier tube and a semi-permeable Teflon membrane across which oxygen in the gaseous or liquid samples stream diffused. The system was of the reversible type since the chemiluminescence signal was nil for samples containing no analyte. If the sample consisted of pure oxygen, the response decayed gradually over a 12-h period; however, if the oxygen concentration was at the usual level for ordinary samples, the same sensing microzone could be used for 2–3 months. The sensitivity to gaseous samples was much higher as a result of the slower mass transfer of O₂ from the liquid to the membrane surface. The sensor features a detection limit comparable to those of conventional sensors for oxygen, but has the advantage of a higher selectivity as EIA does not react with gaseous species with redox properties (*e.g.* Cl₂, H₂S). On the other hand, its most serious practical constraint is the need to operate in the dark.

The flow injection manifold shown in Fig. 5.5.A, which includes a probe-type fluorimetric biosensor accommodated in a thermostated flow-cell at 35°C, was used for the determination of L-glutamate in foods and

pharmaceutical preparations. The active microzone was attached to the end of an optical fibre connected to the detector (see Fig. 2.6.A). Two biosensors

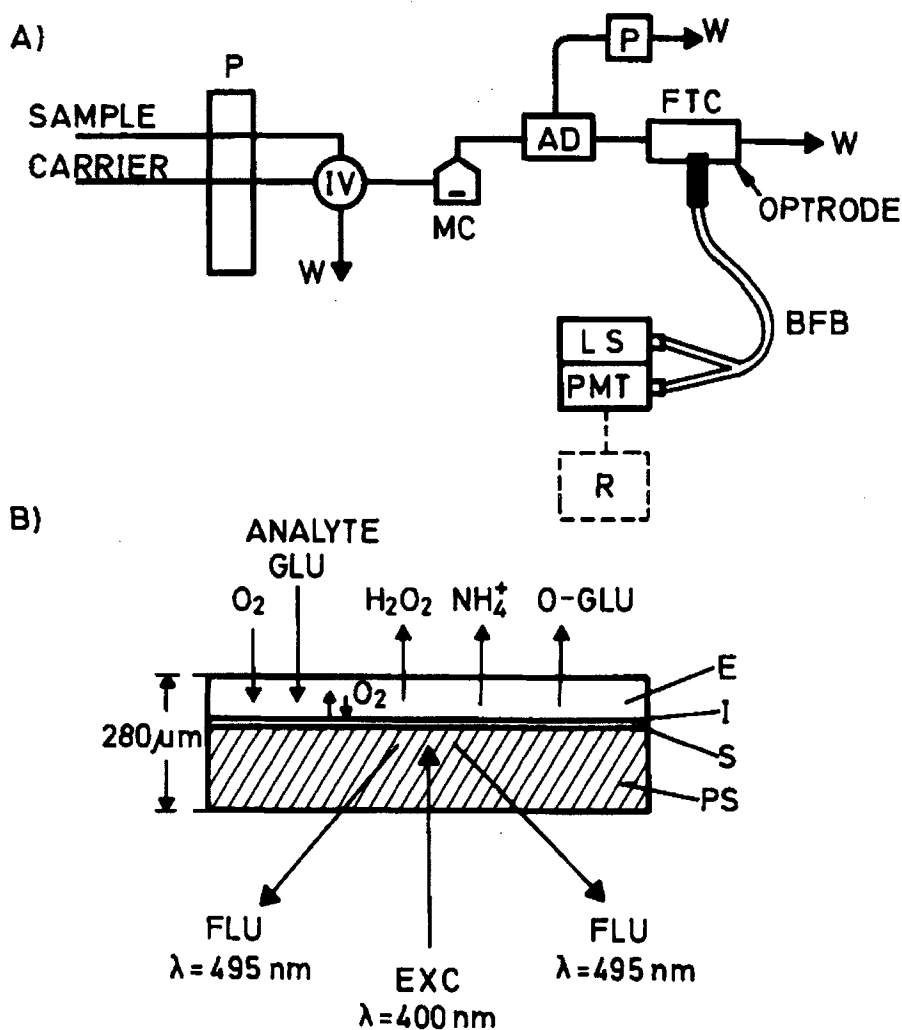
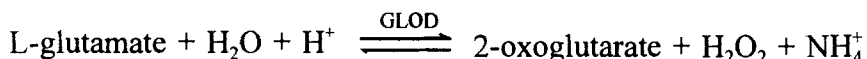


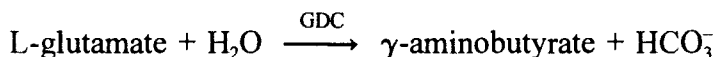
Figure 5.5 — Flow-through biosensor for the determination of L-glutamate. (A) Flow injection manifold. (B) Sensing microzone of the probe sensor (optrode), incorporated in the flow-cell (FTC). P pump; IV injection valve; MC mixing chamber; AD air damper; BFB bifurcated fibre bundle; LS light source; PMT photomultiplier; R recorder; GLU L-glutamate; O-Glu 2-oxoglutarate; E enzyme layer; I optical insulator; S sensing layer; PS polyester support. For details, see text. (Adapted from [6] with permission of Elsevier Science Publishers).

based on two different enzyme reactions were used for comparison. One was based on measurement of the enzyme activity of glutamate oxidase (GLOD):



The carrier used for this purpose consisted of a 0.1 M phosphate buffer of pH 7. The appearance of the sensing microzone is shown in Fig. 5.5.B. The oxygen optrode used was based on a 10- μm silicone rubber film containing dissolved decacyclene as indicator (S) that was fixed on a 110- μm thick polyester support (PS). A 9- μm black PTFE membrane (I) was used for optical insulation. The dye fluorescence was found to be markedly dependent on the concentration of oxygen, which exerted a quenching effect on it. The enzyme (glutamate oxidase) was immobilized on a 150- μm thick immunoaffinity membrane (E). The sensor was prepared similarly as reported by Tretnak *et al.* [7].

The other above-mentioned sensing system relies on measurement of carbon dioxide (rather than oxygen) produced in the biochemical reaction of L-glutamate catalysed by glutamate decarboxylase (GDC):



The carrier used was a 0.1 M phosphate buffer of pH 5. The active microzone was similar to that of an oxygen optrode except for the sensing layer (S), which was a 10- μm thick film of silicone in which an internal buffer including the H^+ ion-sensitive indicator dye (1-hydroxypyrene-3,6,8-trisulphonate, HPTS) dissolved in 10 mM sodium hydrogen carbonate was dispersed. The pH within the micelle-like droplets was found to be altered by carbon dioxide. The optrode construction procedure was described elsewhere [8]. The previous sensor, based on the use of an oxygen optrode and immobilized glutamate oxidase surpasses this carbon dioxide optrode using glutamate decarboxylase in several respects, namely: (a) selectivity (there is no interference from other volatile acids such as acetic acid); (b) stability (the O_2 optrode remains operative for months as opposed to a few weeks); and (c) responsiveness (because oxygen diffuses more readily than does carbon dioxide, the O_2 optrode provides a more rapid response that results in a higher sample throughput).

In 1991, Simon *et al.* [9] reported a flow-through chemical sensor for the determination of ammonium ion in water samples using a sensing

microzone over which a single stream of sample was passed. The microzone included a gas-diffusion membrane and the optrode membrane, which was responsible for sensing proper. The sensor (Fig. 5.6) was placed in the optical path of an ordinary photometer, so it fits the generic configuration shown in Fig. 2.6.C, where the sensing microzone is integrated rather than connected with the detector. The optrode PVC membrane used accommodated a cation (ammonium)-selective neutral ionophore (I), a proton-

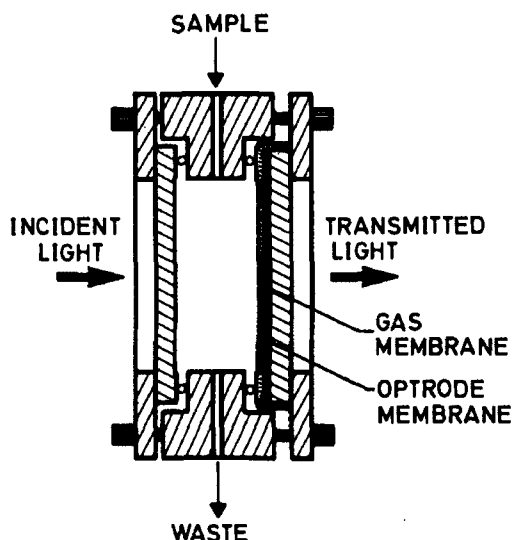
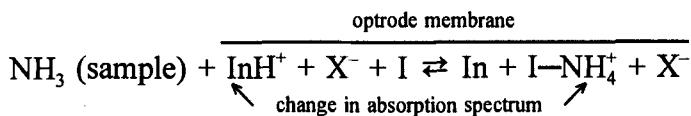


Figure 5.6 — Flow-through chemical sensor for the determination of ammonia. For details, see text. (Adapted from [9] with permission of the American Chemical Society).

selective neutral chromoionophore (In) whose absorption spectrum was altered on protonation and a lipophilic anionic site (X^-) intended to assure electroneutrality of the sensing membrane. The overall sensing process,



involved two steps by which the absorbance at 640 nm was reduced. The features of the sensor were found to depend on the nature of I. Thus, optrodes based on NH_4^+ -selective neutral ionophores of the macrotetrolide

type are more selective than other ammonia derivatives and provide a precision of 4.5% and 2.6% for 10^{-4} and 10^{-3} M ammonia, respectively. Also, valinomycin-based optrodes are moderately selective towards ammonia, but their dynamic linear range is shifted to higher ammonia concentrations, so the reproducibility for samples containing 10^{-4} to 10^{-3} M ammonia is somehow improved (3.2% and 1.6%, respectively). These sensors clearly surpass other ammonia optrodes in selectivity and excel electrochemical ammonia gas sensors in such respects as (a) simplicity (no internal buffer solution is needed), (b) applicability (dry gas-phase samples are equally affordable), (c) miniaturizability, and (d) suitability for qualitative detection by the naked eye. A detailed description of this type of sensor is provided in Section 5.4.2.2.

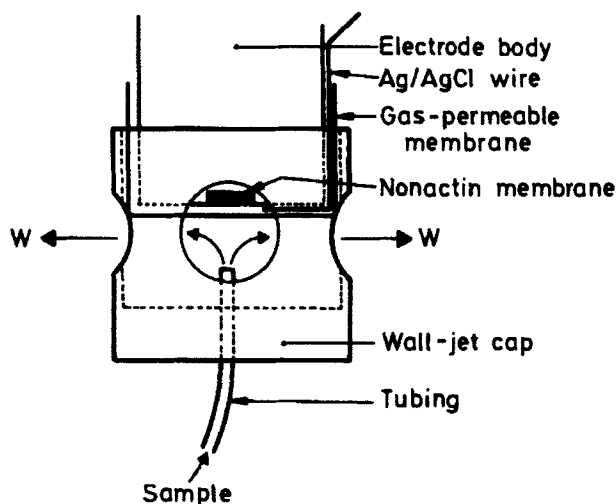


Figure 5.7 — Wall-jet potentiometric flow-through sensor including an internal nonactin based ISE furnished with an outer gas-permeable membrane. For details, see text. (Reproduced from [11] with permission of VCH publishers).

Figure 5.7 shows a typical application of gas-diffusion membranes: isolation of the circulating sample from a voltammetric or potentiometric electrode for the electrochemical determination of gaseous species. The ion-selective electrode depicted in this Figure includes a polymer membrane containing nonactin that is used for the potentiometric determination of ammonia produced in biocatalytic reactions. Interferences from alkali metal ions are overcome by covering the nonactin membrane with an outer hydro-

phobic gas-permeable PTFE membrane including a layer of internal solution to separate the membranes from the miniature reference electrode used. The main use of this sensor is for the determination of species yielding ammonia on enzymatic degradation (*e.g.* creatinine by reaction with iminohydrolase). Because the enzyme reaction yield in the FIA manifold coupled to the sensor is not 100%, the endogenous concentration of ammonia must be taken into account. This shortcoming can be avoided by sequentially injecting two sample aliquots, of which only one is passed through the enzyme reactor, or by using an ion dialysis unit (an ion-exchange Nafion coil bathed in 0.5 M lithium acetate) to remove ammonium ion completely and alkali metals partly prior to passage of the sample through the reactor containing an appropriate immobilized enzyme [11].

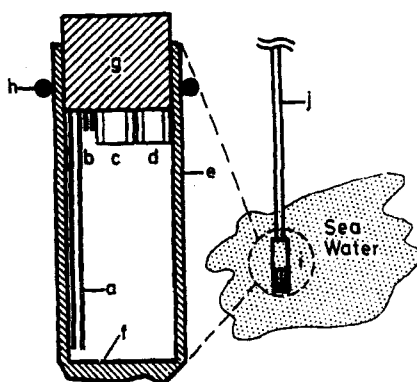


Figure 5.8 — Probe-type sensor based on continuous circulation of a stream containing an acid–base indicator for the batch determination of CO_2 in sea water. (a) Reagent delivery capillary. (b) Reagent exit capillary. (c) Optical fibre from source. (d) Optical fibre to detector. (e) White silicone rubber membrane. (f) White silicone sealant. (g) Epoxy resin. (h) O-ring. (i) Sensor housing. (j) Optical cable. (Reproduced from [12] with permission of the American Chemical Society).

Recently, DeGrandpre [12] developed a probe-type sensor for the determination of $p\text{CO}_2$ in sea water by direct immersion of the probe, which, however, has some connotations of flow-through sensor even though a pH indicator such as Phenol Red ($pK_a = 7.5$) or Bromothymol Blue ($pK_a = 6.8$) rather than the sample is circulated over the sensing microzone—the basic forms of these indicators have a high molar extinction coefficient at 560 and

590 nm, respectively. The sensor consists of an optical microcell with a central cavity through which the reagent is circulated at an appropriate pH and the light input and output optical fibres are placed as shown in Fig. 5.8. The outer silicone membrane used provides a gas-permeable barrier between the circulating reagent and the sea water samples, and also acts as an efficient scatterer for light emitted by the source fibre. This is therefore a continuous, renewable fibre-optic probe-type sensor based on measurement of the light intensity at the absorbance wavelength of the indicator, which is continuously delivered to the fibre tips through capillary tubing. Diffusion of CO_2 from the sea water sample across the sensor walls causes the colour of the internal solution to change. The sensor can operate both in a diffusion-dependent steady state and an equilibrium regime depending on the indicator stream flow-rate used (in the microlitre-per-minute region). The optimal precision is $\pm 0.8 \mu\text{atm}$ for a CO_2 pressure of 300–500 μatm . The response time of the sensor ranges from 11 to 26 min, also depending on the flow-rate used. Its performance was tested on a research cruise; the results were compared with the underway $p\text{CO}_2$ measured simultaneously by using an infrared carbon dioxide analyser.

5.2.2 Sensors based on two sample (donor)–acceptor streams

This type of sensor is schematically depicted in Fig. 5.4.B. The sensing microzone is crossed by two streams rather than one. One such stream acts as the donor and contains the aspirated or injected sample, which is conditioned in order to provide a gaseous reaction product. The other stream acts as the acceptor for the volatile species transferred across the gas-diffusion membrane that isolates the two streams.

These sensors differ from ordinary gas-diffusion units in the fact that the analytical reaction (derivatization of volatile species) and detection take place simultaneously immediately after the continuous separation step. They also differ from (usually electrochemical) sensors using a static internal solution. In this respect, they resemble the sensor reported by DeGrandpe [12], from which they differ in that the sample is not passed through the sensor, even though the reagent is.

Continuous circulation of the acceptor solution, where the sensing process takes place, makes them reusable and hence of practical interest. The acceptor solution may contain the ingredients needed to regenerate the reagent when this is immobilized on the sensing microzone.

There are two possible configurations for this type of flow-through sensor integrating gas diffusion, reaction and detection that differ in whether the reagent is dissolved in the acceptor solution or immobilized on a sensing microzone located near the diffusion membrane. The descriptions below are based on such a difference.

In 1985, Ruzicka and Hansen established the principles behind flow injection optosensing [13–15], which has subsequently been used for making reaction-rate measurements [16], pH measurements by means of immobilized indicators [17,18], enzyme assays [19], solid-phase analyte preconcentration by sorbent extraction [20] and even anion determinations by catalysed reduction of a solid phase [21] —all these applications are discussed in Chapters 3 and 4. Incorporation of a gas-diffusion membrane in this type of sensor results in substantially improved sensitivity (through preconcentration) and selectivity (through removal of non-volatile interferents). The first model sensor of this type was developed for the determination of ammonium [13] and later refined by Hansen *et al.* [22,23] for successful application to clinical samples.

Figure 5.9 shows the (bio)chemical flow-through sensors and on-line coupled continuous-flow configurations used for the determination of creatinine and ammonium ion (A) and urea (B) in undiluted blood serum. The first sensor [22] consists of two black PVC blocks. One of them (*a*) has a single outlet and two optical fibres that intersect the gas diffusion membrane (a 25- μm thick hydrophobic polypropylene film of 0.04 μm pore size) at an angle of 45° and afford reflectance measurements. Through this block, the solution containing the acid–base indicator is circulated. The carrier stream, which transports the injected sample and contains Tris buffer, α -ketoglutaric acid, NADH, EDTA and ADP for maximal activity and stability of the enzyme reactors included in the continuous configuration, is circulated through the other block (*b*). The outlet of this block is used by both the donor and the acceptor stream. The donor stream first impinges on the membrane in the zone where the optical fibres are focussed; this wall-jet arrangement allows the pH change in the acceptor indicator solution to be monitored exactly where ammonia transfer is maximal. The configuration depicted in Fig. 5.9.A2 was designed and optimized for the determination of creatinine, which yields ammonium ion by the catalytic action of creatinine iminohydrolase (CIH). The interference from endogenous ammonium ion in serum is overcome by using a glutamate dehydrogenase (GLDH) reactor,

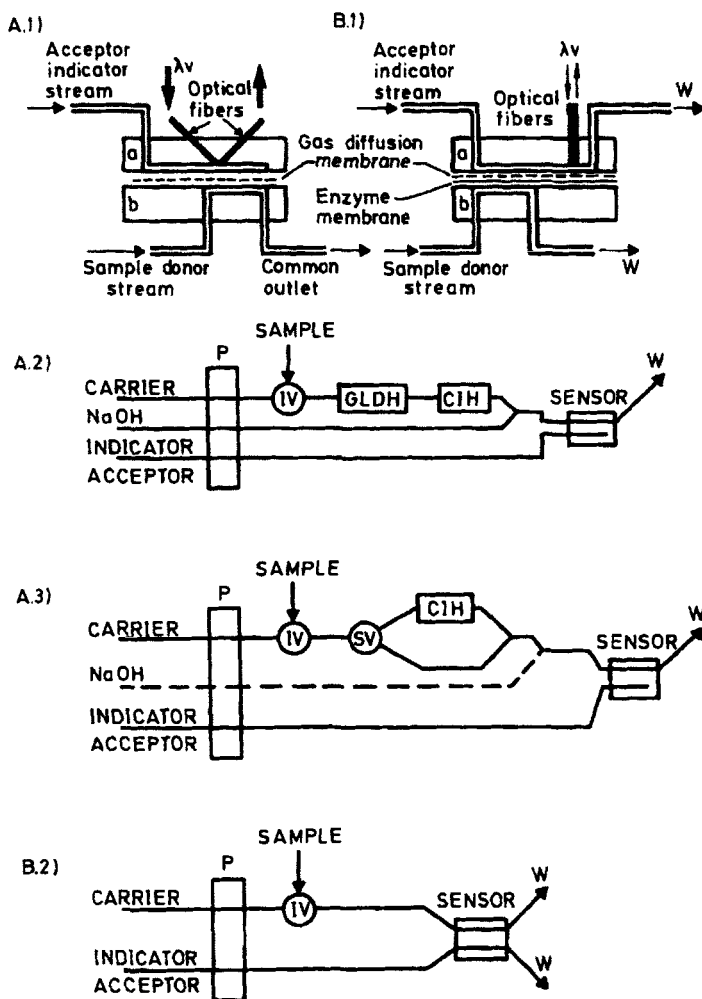


Figure 5.9 — Flow-through sensors based on three integrated processes including gas diffusion where the sensing microzone is crossed by two streams. (A) Determination of creatinine and ammonia in undiluted blood serum: (A.1) diagram of the flow-through sensor used; (A.2) flow injection manifold for the individual determination of creatinine; (A.3) flow injection manifold for the sequential determination of creatinine and ammonia. (B) Determination of urea in undiluted blood: (B.1) diagram of the biochemical flow-sensor used; (B.2) flow injection manifold. GLDH and CIH denote enzyme reactors containing glutamate dehydrogenase and creatine iminohydrolase, respectively. P pump; IV injection valve; SV switching valve; W waste. (Reproduced from [22] and [28] with permission of Elsevier Science Publishers and Marcel Dekker, Inc., respectively).

which, in conjunction with the reaction ingredients, removes the interferent altogether according to



before the NH_4^+ resulting from degradation of creatinine in the CIH reactor starts to be formed. The configuration shown in Fig. 5.9.A3 allows the sequential determination of endogenous ammonium and creatinine. Its functioning relies on the use of a switching valve. The sample is injected and mixed with the carrier, after which it is led to the sensor in the first step. This is followed by passage through the CIH reactor for determining total ammonium, *i.e.* endogenous NH_4^+ plus that resulting from creatinine degradation (the concentration of the latter is obtained by difference). At low analyte concentrations, the analytical signal can be enhanced by stopping the donor and acceptor streams for 16 s at a preset time after injection in order to hold the sample plug in the flow-cell and boost diffusion of ammonia across the membrane. The determination range thus achieved is consistent with the typical concentrations of the two analytes in undiluted blood samples. The good figures of merit of these sensors (sample volume 30 μL ; throughput 60 samples/h; RSD 3%) enable application to real clinical samples with substantial advantages over other continuous methodologies based on potentiometric [23–25], capacitance [26] and spectrophotometric measurements [27].

The sensing microzone of the flow-through sensor depicted in Fig. 5.9.B1 integrates gas-diffusion and detection with two analytical reactions [28], *viz.* (a) the urease-catalysed formation of ammonium ion by hydrolysis of urea (the analyte), which takes place on a hydrophilic enzyme membrane in contact with the sample–donor stream, which contains a gel where the enzyme is covalently bound; and (b) an acid–base reaction that takes place at the microzone on the other side of the diffusion membrane and involves Bromothymol Blue as indicator. This is a sandwich-type sensor including a hydrophilic and a hydrophobic membrane across which the sample stream is circulated —hence it is formally similar to some enzyme electrodes. Since the enzymatic conversion of the analyte must be as efficient as possible, detection (based on fibre optics) is performed after the donor and acceptor streams have passed through the sensor. Unlike the previous sensor (Fig. 5.9.A), this does not rely on the wall-jet approach; in addition, each stream has its own outlet and the system includes two sensing microzones

(one for derivatization and the other for separation). Because this sensor integrates four steps, the continuous configuration needed for its implementation (Fig. 5.9.B2) is simpler than those required by the previous sensors. The sensor allows the direct determination of urea in whole blood with no interference from endogenous ammonium ion, the concentration of which is lower than that produced by urea by several orders of magnitude. The throughput and RSD for this determination are 30 samples/h and less than 2%, respectively. The sensor remains serviceable over at least one week of continuous use, after which measured signals start to degrade somehow. This cannot be the result of the enzyme membrane losing some of its activity since it remains stable for months; rather, it must arise from clogging of the pores of the hydrophobic gas-permeable membrane.

Recently, Ruzicka *et al.* [29] reported a versatile universal sandwich membrane spectrophotometric–chemiluminescence sensor involving gas diffusion or dialysis that combines fibre optics, flow channels, spacers, reflecting surfaces and separation membranes into a robust sensing unit that epitomizes this group’s expertise in sensor development [13–21]. They used it in an arrangement similar to those shown in Figs 5.9.A1 and 5.9.A2 in conjunction with a flow injection manifold for (a) the determination of ammonium ion based on an acid–base indicator reaction taking place in the acceptor stream and photometric detection; and (b) the determination of hypochlorite ion by using an acid donor stream (0.1 M HCl) in order to produce chlorine (the species actually separated) and an acceptor stream containing luminol at pH 12.3 that was stopped for preconcentration. While no application to real samples was reported, the sensor is seemingly quite robust and easy to construct, reconfigure, clean and repair.

5.3 INTEGRATION OF DIALYSIS, REACTION AND DETECTION

Sensors based on integrated dialysis, reaction and detection differ from those described in Section 4.3.1 in the fact that a (bio)chemical reaction takes place after separation (simultaneously with detection). They thus fit the generic configuration depicted in Fig. 5.1.A. Some of the ingredients of such a reaction may be immobilized at the sensing microzone, even though the reaction may also take place in the solution passed through it.

The sample, whether aspirated or injected into a carrier stream, is circulated through these sensors, which can be arranged in two main ways

similarly as those integrating gas diffusion shown in Fig. 5.4. Those in which only the sample stream is passed through are somewhat more commonplace.

The dialysis membrane employed is usually hydrophilic and isolates two aqueous solutions in a static or dynamic regime depending on the particular purpose. While these sensors are formally similar to those discussed in the previous section, it is molecules or ions that are separated (by virtue of a concentration gradient), the process being aided both by the dynamic character of the acceptor solution and the reaction involved, which removes the species transferred across the membrane.

As with sensors based on a triply integrated process involving gas diffusion, there are few reported examples of sensors integrating dialysis, reaction and detection. There follows a description of the most salient examples based on the ingredient of the (bio)chemical reaction that is dialysed at the sensing microzone.

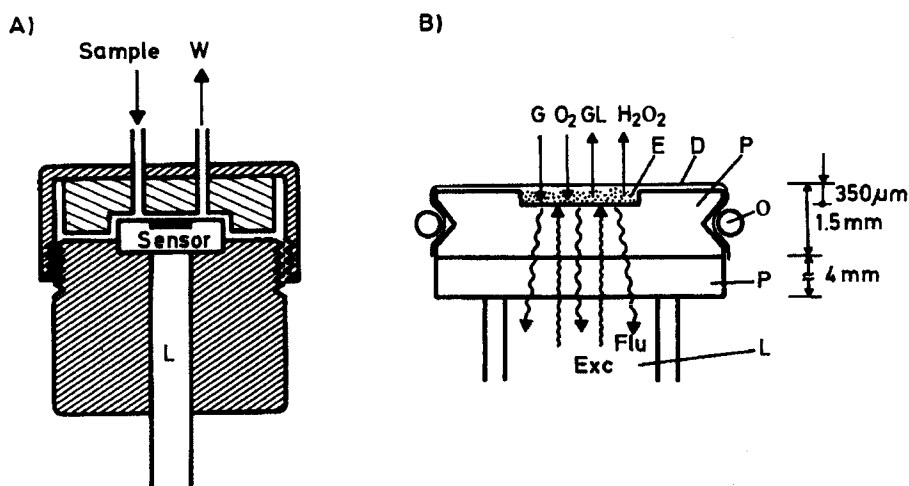


Figure 5.10 — Flow-through sensor based on three integrated processes including dialysis of the analyte for the determination of glucose (GL) in a static solution of glucose oxidase (E) and simultaneous fluorimetric monitoring of its changes by means of fibre optics (L). (A) Flow-through cell. (B) Cross-section of the sensing microzone. P plexiglas; D dialysing membrane; E enzyme solution; O o-ring; L light guide; W waste. Arrows indicate the diffusion processes involved (G glucose, GL gluconolactate). The plate has an OD of 20 mm and a cavity diameter of 4 mm. (Reproduced from [7] and [34] with permission of the Royal Society of Chemistry and Elsevier Science Publishers, respectively).

5.3.1 Sensors based on separation of the analyte

Ruzicka *et al.* [29] used the above-described sensor based on a universal sandwich membrane cell with optosensing for the chemiluminescence determination of phenol in a continuous-flow configuration where the aqueous sample was continuously pumped across one of the membrane sides or circulated ($V_i = 200 \mu\text{L}$) into a distilled water carrier. An also aqueous carrier resulting from continuous mixing of a solution of 4-aminoantipyrine and an oxidant (peroxydisulphate ion) was circulated across the other side of the membrane. On merging with the analyte, this latter stream gave rise to a coloured compound that was detected *in situ* by means of fibre optics. Because the membrane used to separate the two aqueous solutions (Celgard 2500) was hydrophobic and microporous, transfer across it involved a step by which the analyte had to diffuse across the air filling the pores. Accordingly, no dialysis proper took place since the analyte was transferred in the vapour phase. For this reason, the authors claimed that the sensor was based on "membrane separation" instead of dialysis.

Wolfbeis *et al.* [30] developed a flow-through biosensor for the determination of glucose based on the fluorescence changes (intensity rises) resulting from interaction of the analyte with glucose oxidase. The sensor thus required no additional transducer, which substantially simplified the instrumental set-up used for implementation—therefore, this type of sensor does not rely on the usual catalytic effect of an enzyme and monitoring of the oxygen uptake [7], production of gluconic acid (which results in a pH decrease) [31,32] or hydrogen peroxide [33]. The sensing microzone (similar to that depicted in Fig. 2.6.A) was located on the tip of a fibre optic probe that was inserted in a stainless steel flow-cell of $20 \mu\text{L}$ inner volume connected to an ordinary fluorimeter (Fig. 5.10.A). Figure 5.10.B shows a cross-sectional view of the sensing part of the sensor. It consisted of a Plexiglas disk of 20 mm OD and 1.6 mm thickness with a cavity of 4 mm ID, 350 μm depth and 565 μL volume in the middle where a volume of 25 μL of an aqueous solution containing 5 mg of glucose oxidase (GOD) was placed. The mini-chamber was covered with a dialysis membrane that was fixed by means of a rubber O-ring. The membrane was not permeable for the enzyme but allowed the substrates (glucose and oxygen) to diffuse into the reaction microvolume. The enzyme fluorescence changes on interaction with glucose as the associated reduced and oxidized flavins in their free forms (FAD and FADH_2) are known to exhibit different fluorescent properties:



The sensor was regenerated by oxygen in the sample or carrier,



which also diffused, thereby rendering the sensor reversible. The fluorescence changes observed took place over a fairly narrow range of glucose concentration (0.5–0.8 mM). The time needed for a steady-state response to be reached varied from *ca.* 2 to 30 min and the response rate increased with increasing glucose concentration. The baseline restoration time following washing with a buffer solution ranged from 2 to 10 min. Changes in the internal solution pH from 4 to 8.5 had no appreciable effect on the sensor response. The sensor limitations (a narrow determination range and sluggish response) can be lessened by using kinetic measurements. Pumping solutions of glucose at different concentrations through the flow-through sensor for over a fixed interval (typically 65 s), followed by pumping of a buffer solution, resulted in peak heights and integrals proportional to the substrate concentration. Repeatability experiments revealed somewhat poor precision (RSD = 8.1%, *n* = 20). Also, the sensor was applied to no real sample.

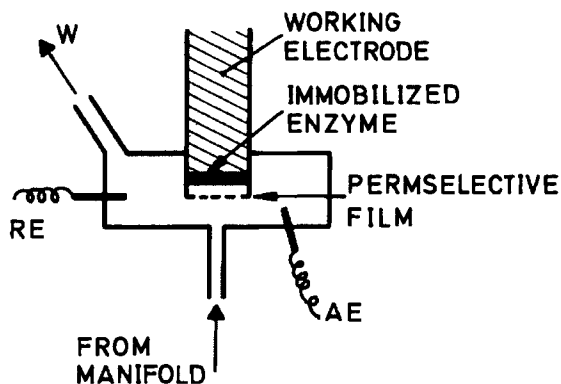


Figure 5.11 — Diagram of a generic electrochemical flow-through biosensor using a permselective film coating the surface of the enzyme electrode. RE reference electrode; AE auxiliary electrode; W waste.

Though less commonplace, there are some electrochemical sensors based on integrated dialysis, reaction and detection. Most of them use a retained enzyme immobilized in a microcavity at the tip of the working electrode that

is covered with a permselective membrane allowing passage of the analyte or substrate but not of potential interferents (*e.g.* macromolecules in biological samples), similarly as described in Section 4.3.1 for flow-through sensors based on integrated separation and electrochemical detection [38]. Figure 5.11 shows a generic scheme for one such sensor using a membrane accommodating an immobilized biocatalyst covered by the dialysis membrane.

5.3.2 Sensors based on separation of the reagent

In 1989, Liebermann *et al.* [34] reported a flow-through sensor in which a fluorimetric reagent (8-hydroxyquinoline-5-sulphonic acid) was forced through an ultrafiltration membrane into the flowing analyte (Mg^{2+}) solution in a special flow-cell furnished with a bifurcated fibre-optic-cable bundle in such a way that reaction and detection took place simultaneously and immediately after separation. The flow-through Plexiglas manifold used included provisions for holding an ultrafiltration membrane of 14 mm diameter with a nominal MW cut-off of 500 Da. The fibre-optic cable was threaded into the manifold block so that the incident beam was aimed at the membrane. The distance from the end of the probe to the membrane could be adjusted from 2 to 22 mm. The analyte solution was pumped through the sensor by means of a variable-speed peristaltic pump. The pressure in the indicator reservoir was controlled via a two-stage regulator connected to a nitrogen cylinder. A prototype probe-shaped sensor in which the membrane was in direct contact with the sample solution was used to test the system response batchwise. The sensor was reversible inasmuch as it responded expeditiously to both increases and decreases in the concentration of dissolved magnesium; in addition it was quite stable, rapid (only 1 s was needed for the maximum signal to be reached and 2 s for the baseline to be restored), sensitive (the detection limit was $50 \mu\text{g Mg}^{2+}/\text{L}$ at a signal-to-noise ratio of 3), and selective (it tolerated the presence of other cations such as Zn^{2+} , Cd^{2+} and Ag^{+} , which also give a fluorescent product with the fluorimetric reagent and are present in sea water at concentrations only five times lower than that of the analyte). This pressurized membrane indicator-based sensor offers some advantages over immobilized fluorigenic indicators used in conjunction with fibre-optic chemical sensors, namely: (a) increased responsiveness and reversibility arising from continuous renewal of the indicator reagent at the end of the fibre-optic probe; (b) minimal liability to problems arising from "poisoning" of the sensing microzone by sorbed

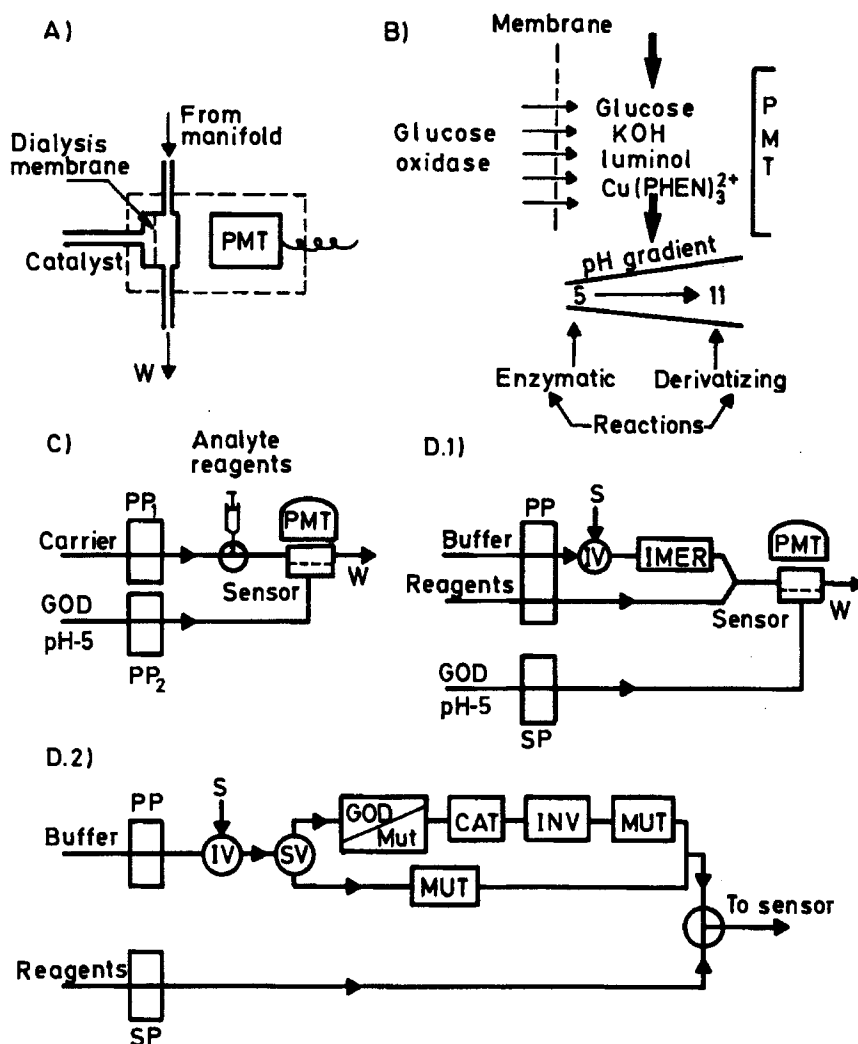
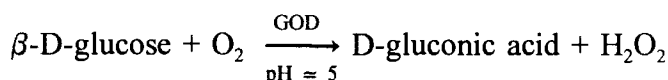


Figure 5.12 — Flow-through biochemical sensor based on three integrated processes including dialysis of the catalyst for the determination of glucose and sucrose. (A) Schematic diagram of the sensing microzone. (B) Formation of a pH gradient between the membrane surface and the PMT (photomultiplier detector). (C) Continuous-flow injection manifold for the determination of glucose: (D.1) manifold for the determination of sucrose (IMER, immobilized enzyme reactor containing invertase and mutarotase); (D.2) manifold for sucrose determination using several IMER columns (GOD glucose oxidase; MUT mutarotase; CAT catalase; INV invertase) via destruction of original glucose (upper channel) and sucrose measurements (lower channel). S sample; PP peristaltic pump; SP syringe pump; IV injection valve; W waste. (Reproduced from [35] and [36] with permission of the American Chemical Society).

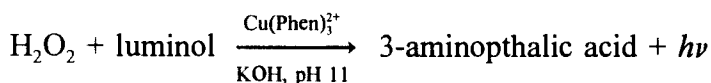
foreign substances, which is a frequent occurrence with natural waters; (c) improved long-term stability (in practice, the sensor service life could be extended indefinitely by fitting the probe with a renewable indicator/membrane unit).

5.3.3 Sensors based on separation of the catalyst

Niemann *et al.* [35,36] developed a series of chemiluminescence biosensors based on two different principles (see Figs 5.12.A and 5.12.B) for integrated separation, reaction and detection. A microporous membrane consisting of polypropylene of 0.4 μm pore size coated with a non-ionic surfactant for improved wettability, and nylon 66 of 0.2 μm pore size was placed in the flow-cell to force the enzyme (glucose oxidase, GOD) at a flow-rate of 3.5 $\mu\text{L}/\text{min}$ into a stream containing the analyte and the other ingredients for the chemiluminescent reaction that was passed through the flow-cell at a rate of 10 mL/min . A pH gradient between the membrane and the detector (PMT) was thus created. This provided an appropriate pH for the two reactions taking place at the sensing microzone, namely (a) the GOD-catalysed oxidation of the analyte (glucose) at pH 5 near the membrane,



and (b) diffusion of hydrogen peroxide in the sample–reagent stream, where it gave rise to a classical luminescence reaction that proceeds optimally at pH 12, catalysed by the Cu(II)–phenanthroline complex:



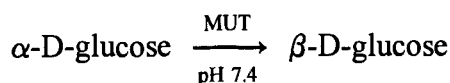
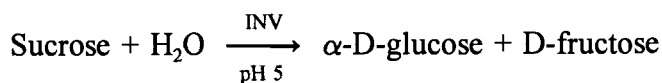
Therefore, this sensor integrates a biochemical and a chemical reaction with a prior separation (dialysis) and chemiluminescence detection. The process involves the following steps: (a) dialysis of the enzyme; (b) enzymatic oxidation of the reagent; (c) derivatization of hydrogen peroxide; and (d) detection of the chemiluminescence produced. Such an original approach offers several advantages over similar methodologies, namely:

- (a) The sample integrity is preserved throughout the process and contamination by the reagent is minimal;

- (b) The volume of catalyst solution required is only a few microlitres per assay, which is extremely important when using enzyme solutions;
- (c) Light emission is localized near the microporous membrane;
- (d) The two reactions involved can be sequentially implemented in an expeditious manner at markedly different pH values thanks to the pH gradient established;
- (e) The catalyst and analyte are mixed very rapidly under controlled conditions with no need for stirring; and
- (f) The sensor is quite straightforward and inexpensive.

Figure 5.12.C shows the on-line coupled configuration used with this sensor for the determination of glucose in serum [35]. The sample was injected into a carrier containing 2×10^{-4} M luminol, 2×10^{-5} M Cu(II)–phenanthroline complex and 0.5 M KOH. On the other hand, the enzyme solution contained biphthalate buffer of pH 5.0. The modifier concentrations were chosen in such a way as to create the above-mentioned pH gradient. The rise time for the peaks was *ca.* 1 s, the peak width at half-height 8–10 s, and the baseline decay time 20–30 s. Because the enzymatic reaction was much faster (0.5 ms half-life at pH 5) than the solution residence time in the cell, all of the injected glucose was probably converted to hydrogen peroxide. A log–log plot of chemiluminescence intensity was linear over the range 2×10^{-7} to 2×10^{-5} M, the detection limit for glucose being 5×10^{-8} M. The precision was 2–3% as RSD. The results obtained in the determination of glucose in serum were consistent with those provided by a Beckman Glucose Analyzer 2.

The continuous configurations depicted in Figs 5.12.D1 and 5.12.D2 were designed by Nieman's group for application of this sensor to the determination of sucrose (and glucose) in soft drinks, breakfast cereal and cake mix [36]. The analyte is converted into β -D-glucose, to which the sensor is responsive, in two reaction steps that are catalysed by invertase (INV) and mutarotase (MUT):



Both enzymes are immobilized on controlled pore glass and placed in the IMER included in the configuration of Fig. 5.12.D1. The carrier, which is circulated at a flow-rate of 1 mL/min, is adjusted to an intermediate pH 6 in order to facilitate establishment of the optimal pH values for the enzyme reactions and provide a basic medium on merging with the reagent stream (luminol + copper chelate + KOH). The GOD solution (pH 5) is introduced into the sensor at a rate of 5 μ L/min by means of a syringe pump. The linear determination range and RSD thus achieved for glucose are 5 μ M–1 mM and 2–3%, respectively. The results compare favourably in terms of throughput, simplicity and sensitivity with those provided by other existing alternatives.

One serious problem encountered in applying the sensor to the determination of sucrose in foodstuffs lies in the glucose they contain. This shortcoming was circumvented by designing, optimizing and using the configuration shown in Fig. 5.12.D2, which allows one not only to remove native glucose, but also to determine it in the same sample by sequential injection of two aliquots and the use of a switching valve located immediately after the injection valve. Glucose is determined by passing the injected sample stream through the channel containing mutarotase only; hence this manifold is similar to that shown in Fig. 5.12.C with the exception that glucose is wholly converted to its β form, to which the sensor is responsive. As the switching valve is actuated, a new sample aliquot is injected that is successively passed through several enzyme reactors intended to (a) convert all endogenous glucose to hydrogen peroxide by effect of glucose oxidase and mutarotase; (b) decompose the H_2O_2 produced with the aid of catalase; (c) convert sucrose into α -D-glucose (with invertase as the catalyst); and (d) transform all the glucose from sucrose into β -D-glucose by effect of mutarotase. The configuration was successfully used for the sequential determination of both analytes in foodstuffs.

An experimental set-up such as that depicted in Fig. 5.12.C was also used by Nieman's group for the determination of free and total cholesterol in serum [37]. A solution of the enzyme (cholesterol oxidase) at pH 7 was forced through a microporous membrane. The pH gradient across the membrane (from 7 to 9) facilitated the enzymatic degradation of the analyte and subsequent diffusion of the hydrogen peroxide formed to the PMT in the reagent/sample stream in order to react with luminol in the presence of horseradish peroxidase as the catalyst at pH 9 —the copper chelate commonly used as catalyst for this purpose requires pH 11. The system per-

formance was fine-tuned by optimizing the operational conditions. To this end, Triton X-100 was tested both to disrupt serum lipoprotein complexes and hold free cholesterol thus released in the micellar solution in a form that was suitable for enzymatic oxidation. The determination of total cholesterol required cholesterol sterase and sodium cholate to be added to the sample (both were omitted for determining free cholesterol). The sample was deproteinated by precipitation with Somogy's reagents (barium hydroxide and zinc sulphate). After centrifugation, the supernatant was mixed with the chemiluminescent reagents and Triton X-100 prior to injection into the continuous configuration coupled to the sensor. The most salient assets of this methodology are quite a high throughput (60 samples/h) and economical usage of the enzyme (0.01 units per assay).

5.4 INTEGRATION OF SORPTION, REACTION AND DETECTION

Flow-through sensors involving a sorption process (Fig. 5.2.3) are the most commonplace of all based on a triply integrated process because those relying on gas diffusion and dialysis (discussed in Sections 5.2 and 5.3, respectively) are only applicable to given types of transferred analytes or reagents and because of the wide variety of synthetic materials available for aiding sensing in a process involving separation, reaction and detection.

Most of these sensors use a permanently immobilized reagent at the sensing microzone (Fig. 5.3) that plays a twofold role, *viz.* (a) retaining the analyte by sorption in one of the many ways in which a species can be transferred from a solution to an active solid (or *vice versa*), and (b) inducing a change (*e.g.* in colour or luminescence) that can be detected by the integrated or connected detection system used. Some of these sensors use no immobilized reagent, though. In fact, a few are based on transient retention of the analyte or its reaction product, followed by a chemical reaction in the solid state on passage of a stream containing the reagent. Still others are based on retention of a reaction product generated elsewhere in the sensing microzone.

Most of these sensors fit the general configuration shown in Fig. 5.1.C, *i.e.* the simultaneous sorption, reaction and detection involved cannot be resolved in space or time. However, separation occasionally takes place in the same zone, but sequentially, before (type A) or after (type B) the (bio)chemical reaction, whereas detection is simultaneous with the reaction or separation, respectively.

One other classification of this type of sensor is based on functioning, which is determined by the features of the overall sensing process, according to which there are (a) fully reversible sensors (*viz.* those that require no regeneration, in which the sensing microzone preserves its integrity and responds accurately to changes in the analyte concentration in the sample after many determinations); (b) irreversible–reusable sensors (*viz.* those that involve two steps —usually retention and elution— and hence require a sample and a regenerating stream to be sequentially passed over the sensing microzone); and (c) *quasi*-reversible sensors (*viz.* those in which the sensing microzone retains and accumulates the analyte permanently and are thus serviceable for a limited number of determinations).

The sorbent materials used to construct this type of sensor are widely varied (ion exchangers, adsorbent solids, polymers) and are employed as particles (larger than 30 μm in order to avoid overpressure in the flow system) or films. Most of these sensors are optical and rely on absorption, reflectance or molecular fluorescence measurements. In order to ensure that the sensing microzone is fully compatible with the detector, the sorbent material used must be as transparent as possible (photometry) or give rise to no appreciable light scatter (fluorimetry) so that the baseline (resulting from passage of the carrier) may be as low as possible.

Some of the flow-through sensors integrating reaction and detection discussed in Chapter 3 could also have been included here since their functioning somehow involves a separation (retention by sorption). Most immunoassays involve retention/elution in the formation/destruction of a complex when one of the reaction ingredients (the antigen or antibody) is retained by the solid matrix of the sensing microzone. Section 3.3 describes a number of flow-through immunosensors based on optical (mainly luminescence) and electroanalytical (amperometric, potentiometric and capacitance) transducers. Those sensors using an acid–base indicator immobilized on the sensing microzone (Section 3.5.1.1) could also be included here inasmuch as the analyte (H^+ ion) has to be retained and released for sensing. Other, not explicitly mentioned examples show that the classification used to deal with the material exposed in this book is not flawless. In fact, many of the flow-through sensors discussed in Chapters 3–5 share some traits and could therefore have been ordered and dealt with in a different way.

The descriptions of sensors integrating sorption, reaction and detection provided below are classified according to the type of immobilization

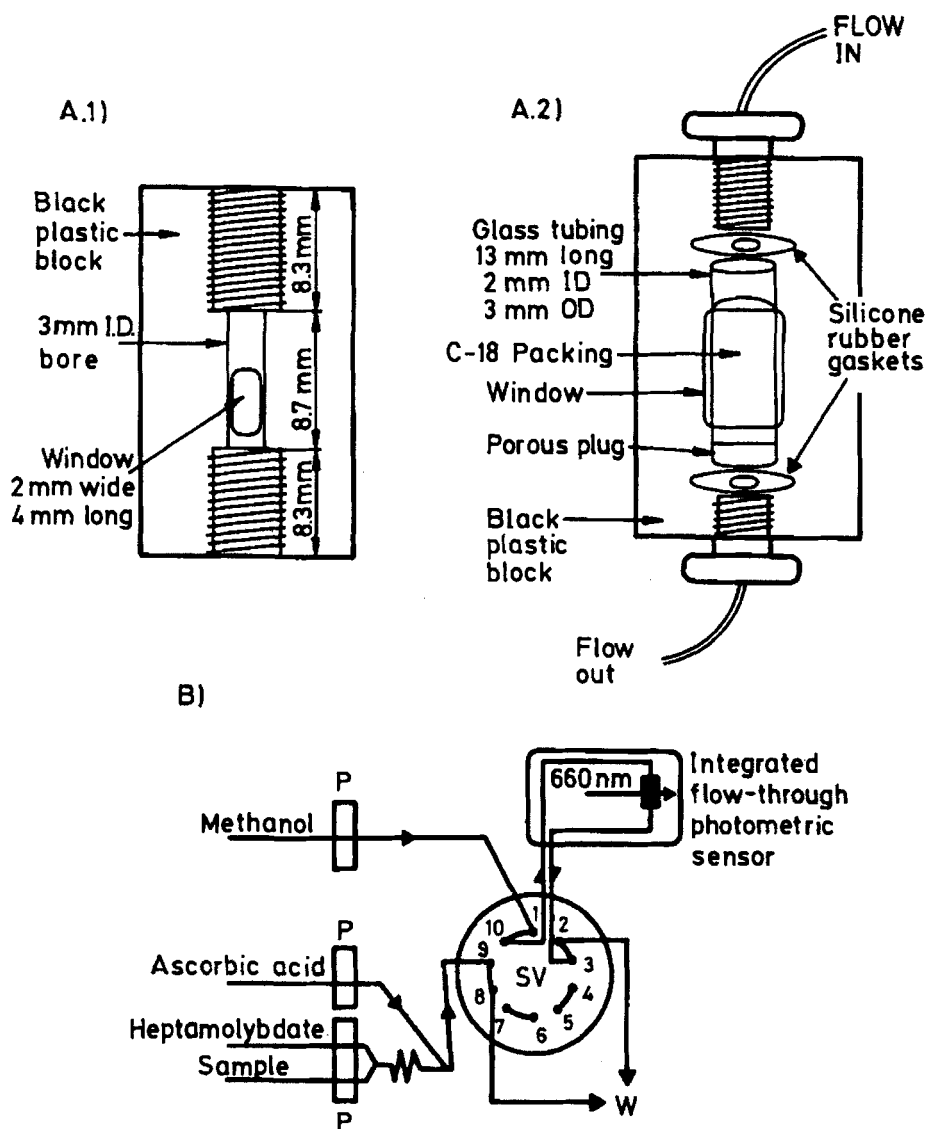


Figure 5.13 — Irreversible-reusable flow-through sensor for the kinetic multidetermination of phosphate and silicate based on integrated sorption of a reaction product, reaction (*in situ* reduction) and photometric detection. (A) Microsensor block (1) and components (2). (B) Continuous-flow configuration coupled on-line to the sensor. P peristaltic pumps; SV switching valve; W waste. For details, see text. (Reproduced from [39] with permission of the American Chemical Society).

(transient or permanent) used to retain either the reagent or product of the (bio)chemical reaction involved.

5.4.1 Sensors based on transient immobilization of a reaction product

There are few sensors of this type, which, however, are as interesting as the previous ones. Unlike other sensors, the sensing microzone contains no immobilized reagent even though it may host a derivatizing reaction. The

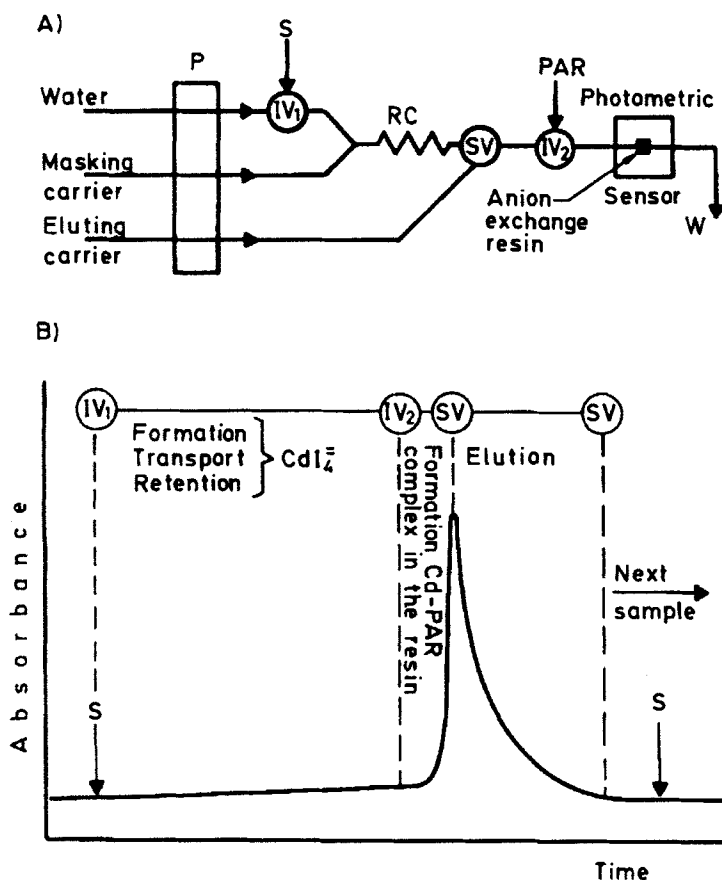


Figure 5.14 — Flow-through sensor for the determination of cadmium at the nanogram per millilitre level based on integrated sorption, reaction and detection. (A) Flow injection configuration including the photometric sensing unit. (B) Typical absorbance–time profile showing the processes occurring in A. IV, injection valve; SV switching valve; P pump; S sample; RC reaction coil; PAR solution of the chromogenic ligand pyridylazoresorcinol; W waste. For details, see text. (Reproduced from [40] with permission of Marcel Dekker, Inc.).

three sensors described below are rather different from one another and open up interesting prospects.

In 1990, Ruzicka *et al.* [39] developed a flow-through sensor based on the formation of two heteropoly acids in an automatic continuous-flow system, their retention at the sensing microzone and the subsequent *in situ* formation of Molybdenum Blue by passing an ascorbic acid stream simultaneously with photometric detection. The system fits the configuration depicted in Fig. 5.1.A, where separation takes place before the reaction and detection, which are simultaneous. The sensor is of the irreversible-reusable type as the reaction product must be eluted before the next sample is processed. Its sensing microzone is integrated with the spectrophotometric detector used (Fig. 2.6.C). Figure 5.13 shows both the sensor (A) and the continuous configuration coupled to it (B). The sensing microzone consists of C_{18} silica beads that are packed in a glass cylinder stoppered by a porous glass plug at one end. All other elements are shown in detail in Fig. 5.13.A. The sensor lightpath length, 2 mm, ensured that the sorbent material was fully transparent. The continuous configuration uses a sample stream that is continuously introduced by aspiration (so as to facilitate preconcentration) and merged with a heptamolybdate stream to form the heteropoly acid in the coil; the product is retained quantitatively by attaching the coil directly to the sensor via the 10-way valve shown in Fig. 5.13.B. Later, an ascorbic acid stream is introduced and insertion of sample and reagent is stopped by actuating the peristaltic pumps in order to implement the derivatizing reaction in the sorbent, which takes place simultaneously with photometric detection at 600 nm. Next, a methanol stream is inserted by actuating the switching valve so as to elute the reaction product. This approach was applied to the simultaneous determination of phosphate and silicate at the microgram and milligram per millilitre level, respectively, by using a differential kinetic method based on differences in the reduction rate between the two heteropoly acids. Data were analysed by partial least-squares regression and errors of prediction of *ca.* 10% were obtained for both components.

Richter *et al.* [40] developed a flow-through sensor integrating sorption, reaction and detection for the determination of cadmium also based on the configuration depicted in Fig. 5.1.A (*i.e.* with separation taking place prior to simultaneous reaction and detection). Figure 5.14 depicts the continuous-flow configuration coupled on-line to the sensing microzone, which was integrated in a flow-cell of 1.5 mm pathlength packed with QAE-Sephadex

anion-exchange resin and placed in an ordinary photometer. The sensor resembles those described in Section 4.3, even though it involves an *in situ* reaction at the sensing microzone. The sample containing the analyte (Cd^{2+}) is injected via valve IV_1 into an aqueous stream that is subsequently merged with another containing masking ligands (thiosulphate, tartrate, fluoride and iodide) to form the anionic complex CdI_4^{2-} in the reaction coil (RC), the complex being immediately retained at the sensing microzone. Injection of a microvolume (100 μL) of 0.1% pyridylazoresorcinol (PAR) via the other injection valve (IV_2) causes the absorbance at 540 nm to increase on passage through the sensor in response to the formation of the Cd(II) –PAR complex by displacement of the iodide complex in the exchanger. A stream of eluting–regenerating carrier containing EDTA, ammonium nitrate and potassium iodide is subsequently passed by actuating the switching valve; in this way, Cd^{2+} is fully removed from the sensing microzone and converted into cadmium iodide. As a result, the baseline is restored and the sensor made ready for processing the next sample. The determination limit and throughput thus achieved are 8.4 ng/mL and 25 samples/h, respectively. Despite the low selectivity of the chromogenic reaction, the sensor is acceptably selective towards cadmium thanks to the prior formation of the CdI_4^{2-} complex, which is retained (thereby excluding those cations that do not form an iodide complex under these conditions), and the addition of tartrate, which prevents precipitation of basic salts.

One other representative example of this type of flow-through sensor (Fig. 5.1.A) is a biosensor developed by the authors' group. It is based on the immobilization of both the catalyst and the reaction product, which are retained on suitable materials packed in a photometric flow-cell, though in a different way: the catalyst is immobilized permanently for repeated use, whereas the reaction product is retained temporarily for monitoring, after which it is discarded [41]. The packed flow-cell used is shown in Fig. 5.15.A. It contains two layers of packing material: an upper layer of packed controlled pore glass containing an immobilized enzyme, and a second layer, located in the cell lightpath, consisting of an exchange resin for retaining the reaction product. Two filters prevent sweeping of the packed material from the flow-cell. The mechanism for retention/reaction and elution in the flow-cell is illustrated in Fig. 5.15.B. The substrate molecules enter the flow-cell and undergo the enzymatic reaction on reaching the catalyst (first layer). The product thus formed is retained and concentrated on passage through the resin (second layer), after which it is removed by using an appropriate

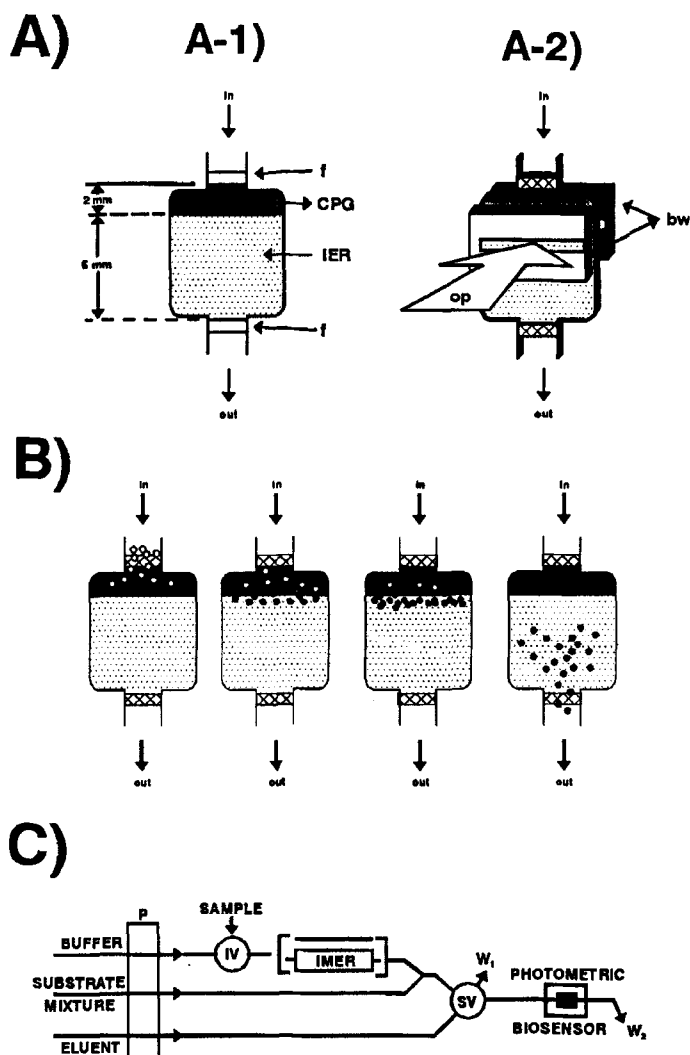


Figure 5.15—Flow-through optical biosensor based on permanent immobilization of the enzyme and transient immobilization of the reaction product. (A.1) Sensing microzone. (A.2) Limited irradiated zone for enhanced sensitivity. (B) Processes occurring in the flow-cell. (C) Flow injection manifold coupled on-line to the biosensor, including or excluding an immobilized enzyme for the determination of glucose and hydrogen peroxide, respectively. CPQ controlled pore glass beads containing peroxidase; IER ion-exchange resin; f filters; op optical path; bw black wall; P pump; IV injection valves; SV switching valve; IMER immobilized enzyme reactor; W waste. For details, see text. (Reproduced from reference [41] with permission of the American Chemical Society).

eluent. The enzymatic reaction used is the peroxidase-catalysed oxidative condensation of 4-aminophenazone and an aniline derivative in the presence of hydrogen peroxide, which yields an alkylaniline cationic derivative that is retained in the upper part of the Sephadex carboxymethyl resin. The organic cation is subsequently removed by passage of an eluting stream of sodium chloride.

Figure 5.15.C shows the auxiliary manifold used for application of this sensor to the determination of hydrogen peroxide. The sample is injected into an appropriate buffer that is merged with a stream of substrate. The mixture then reaches the biosensor, where the enzymatic reaction and retention of the product formed take place. On switching the valve, the eluting stream flushes the reaction product retained in the biosensor, which is thus made ready for the next sample. In this way, H_2O_2 can be determined at the parts-per-billion level over a wide linear concentration range with excellent reproducibility.

The scope of application of this biosensor can be dramatically expanded by including an oxidase reactor immediately after the injection valve in the continuous system. The hydrogen peroxide formed in the oxidase-catalysed reaction can be merged with the most suitable buffer for the next enzymatic step to take place in the flow-cell in contact with peroxidase immobilized on CPG. Both photometric and fluorimetric monitoring are possible depending on the nature of the substrate for the second enzymatic step. This biosensor was used for the determination of glucose in serum by use of a glucose oxidase reactor. The procedure used was similar as that for the previous application, *i.e.* it essentially involved injection of the serum sample after appropriate dilution and conversion of glucose into β -D-gluconic acid and hydrogen peroxide, the latter of which reacted with the reagent mixture on passage through the peroxidase-loaded CPG layer packed in the flow-cell. This method for the determination of glucose also has excellent features including a wider linear range and a good relative standard deviation. Its performance was assessed by applying it to the determination of glucose in various serum samples. The results were quite consistent with those provided by the standard recommended method. The recovery, as checked by using two additions of standard to aliquots of each sample, was also excellent (between 97% and 102% in all instances).

5.4.2 Sensors based on permanent immobilization of the reagent

In this type of sensor, the reagent immobilized at the sensing microzone plays a twofold role by retaining the analyte and giving rise to a detectable derivative. The detector used can be connected to (*e.g.* via fibre optics) or integrated with the sensing microzone. A general distinction can be made according to whether the reagent used is directly formed by derivatization of the analyte or requires an additional process involving an ionophore. This criterion is followed in describing the most representative examples below.

5.4.2.1 Sensors based on direct immobilization of a single reagent

In this type of sensor, a chromogenic or fluorogenic reagent is immobilized in the sensing microzone, which can be integrated with the photometer or fluorimeter or connected to it via fibre optics. The reagent can be immobilized on a solid (*e.g.* by adsorption or chemical linkage) or a transparent membrane at some stage of the preparation process. A clear distinction should be made at this point between reversible and reusable sensors, which are often confused. As a rule, this type of flow-through sensor is of the irreversible-regenerable type. Regeneration (elution of the sorbed analyte and readying of the sensing microzone for processing the next sample) is accomplished by passing a stream of reagent or a mixture. Figure 2.13 shows the continuous-flow configurations typically used in on-line coupling with this type of sensor. Some have been used for the determination of metal ions by means of ligands immobilized in the sensor, as well as for quantitation of other species including water and sulphur dioxide by unusual reactions. Below are described some of the more representative examples according to the type of detector (photometric, fluorimetric, reflectance) used.

Lázaro *et al.* [42] reported the first flow-through photometric sensor of this type based on retention of 1-(2-pyridylazo)-2-naphthol (PAN) by adsorption on commercially available low-colour cation-exchange resin (Dowex 50-W) packed in a flow-cell of 40 μL inner volume for the determination of copper. The cell was not fully packed with exchanger so as to avoid degraded sensitivity (the packing top lay just above the lightpath, so no analyte was retained in the "dark" zone). Detection in an ion-exchange phase had previously been used in manual procedures for measuring absorbance [43], reflectance (by means of fibre optics) [44] and fluorescence [45]. Despite its practical limitations, the sensor was used as a model for subsequent developments. Figure 5.16 shows the integrated sensing microzone and the two on-line coupled continuous configurations used. The carrier employed

in both contained a chelating ligand (thioglycollic acid, TGA) for expeditious flushing of complexed copper from the sensing microzone, a small amount of PAN ($10^{-4}\%$) in order to offset the loss of immobilized ligand on continuous passage of the stream through the sensor, and sodium chloride for faster regeneration. In the flow injection mode (Fig. 5.16.A), an unusually large sample volume ($0.5\text{--}1.0\text{ mL}$) was injected directly into the eluting carrier — the manifold is suitable for high analyte concentrations ($0.1\text{--}26\text{ }\mu\text{g/mL}$). In the continuous-flow mode (Fig. 5.16.B), the switching valve allows the sample and carrier to be introduced sequentially into the system. This latter configuration allowed implementation of the kinetic concentration method (see Fig. 2.19.B1) based on measurements of the slope of the rising portion of the signal over the first 60 s. The figures of merit of the method (detection limit 1 ng/mL ; linear range $5\text{--}130\text{ ng Cu/mL}$; RSD 3%, throughput 50 samples/h) and its low sample consumption testify to the great assets of this novel methodological approach.

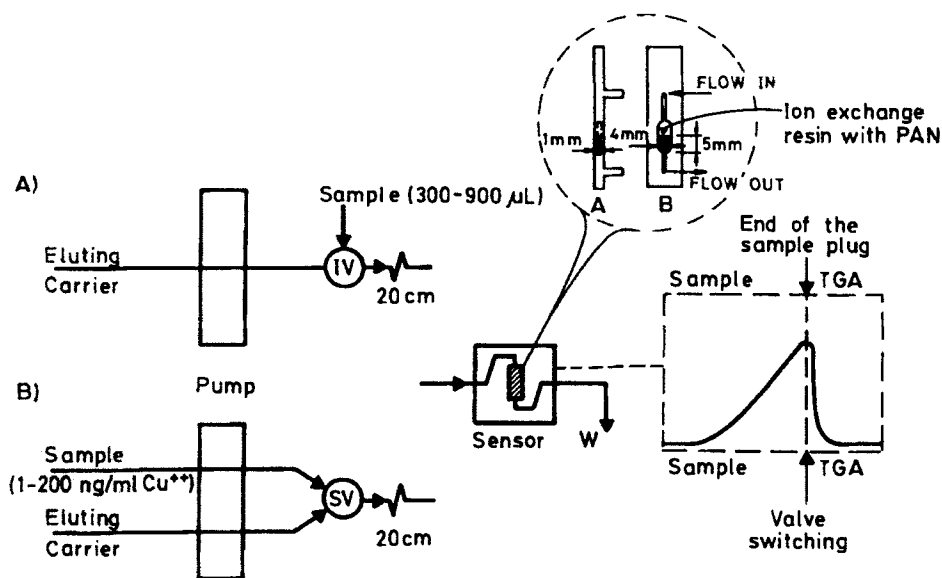
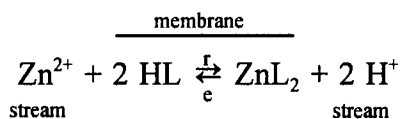


Figure 5.16 — Flow-through photometric sensor for the determination of traces of copper based on the immobilization of a chromogenic ligand (PAN) in a special flow-cell coupled on-line with a flow injection (A) or continuous-flow (B) configuration. IV injection valve; SV switching valve; W waste; TGA thioglycollic acid. For details, see text. (Adapted from [42] with permission of Elsevier Science Publishers).

Iron(III) can be determined at the nanogram per millilitre level by using a similar approach and the configuration of Fig. 5.16.A. Unlike the previous one, it uses an anion exchanger (Dowex 1-X2-200) in the sensing microzone in order to retain thiocyanate (the derivatizing reagent); also, the eluting carrier, containing alkaline fluoride, EDTA and acetate as masking agents, does not include the derivatizing reagent (instead, a stream of this reagent containing 0.4 MM ammonium thiocyanate in 0.05 M HCl, is merged with the carrier prior to the integrated photometric sensor). The Fe(III)–SCN[−] complex thus forms in the resin, which is maintained in its thiocyanate form throughout the process. After the signal peaks, the carrier regenerates the sensor as the masking agents in it remove the iron on passage of the trailing end of the sample plug. This method is quite selective and sensitive (detection limit 3 ng Fe/mL), and features a wide linear determination range (10–400 ng/mL), so it can be used for the determination of iron traces in natural waters and wine, with average recoveries of 100.3–98.8%, a precision of 1.6–2.5% as RSD, and the need for no sample pretreatment [46]. It is interesting to note that such a classical, though obsolescent reaction on account of its poor performance in the photometric determination of iron, is quite fit for use in this sensing approach by virtue of the *in situ* enhanced sensitivity resulting from concentration of the reagent at the sensing microzone and avoidance of the adverse effects of the reagent instability by use of highly expeditious measurements.

Simon *et al.* [47] developed a photometric flow-through sensor for the determination of zinc using a transparent PVC membrane accommodating a lipophilized ligand, *viz.* 1-octadecyloxy-4-(2-pyridylazo)resorcinol, a water-insoluble derivative of PAR. The mechanism by which the analyte is retained in the membrane and eluted from it is similar to a liquid–liquid extraction



and involves exchange of the cation by two protons, which assures electro-neutrality in the membrane. The constant for this heterogeneous equilibrium depends on the formation constant of the complex and the distribution coefficients of the ions (Zn²⁺ and H⁺) between the aqueous (stream) and organic phase (membrane). This optrode membrane exhibits a longer response time (5–6 min) than similar membranes accommodating neutral ionophores (3–4 min) developed by Simon *et al.* which are described in the

following section. The slower response of the membrane is a result of the complex formation and dissociation involved in the zinc sensor being somewhat slow relative to diffusion, on which the response time of ionophore-based optrodes essentially depends. The sensor design is similar to that depicted in Fig. 5.6 except for the fact that no gas-diffusion membrane is used [10]. The authors failed to describe the continuous-flow configuration they used in conjunction with the sensor, through which the analyte and HCl streams were alternately circulated (the sensor is thus of the irreversible–reusable type). The system allows the determination of zinc over the range from 1×10^{-6} to 3×10^{-3} M. The high stability of the membrane prevents appreciable losses of the lipophilized reagent. On the other hand, the lack of specificity of the ligand results in poor selectivity.

Morin immobilized by adsorption in Dowex 1-X4-1000 anion-exchange resin was used to construct a flow-through fluorimetric sensor for the determination of beryllium traces [48]. The modified material was packed in a commercially available fluorescence flow-cell of 25 μ L inner volume and 1.4 mm pathlength than was placed in an ordinary spectrofluorimeter. The continuous configuration used for implementation of this sensor, similar to that of Fig. 5.16.A, consisted of a single stream of transporting–eluting carrier (0.2 M HNO_3) containing a small amount of morin (20 ng/mL) in order to offset its gradual desorption from the sensing microzone. The sample (1 mL) containing EDTA was injected into this dual-purpose stream and transferred to the sensor, where it gave rise to a transient signal. The sensor was then rapidly regenerated by the carrier after the sample was passed. The method thus developed features a linear range of 1–40 ng/mL, and RSD of 1.7% and a throughput of 30 samples/h. However, its most salient asset is its high selectivity (the tolerated Be-to-foreign ion ratios for many elements including calcium, copper, cobalt, iron, magnesium and aluminium are higher than 1:100, in clear contrast with the serious interferences of these ions with the manual method). Such a high selectivity makes the method applicable to a wide variety of metallurgical samples.

Figure 5.17 shows two fluorimetric flow-through sensors for the determination of metal ions using an immobilized ligand for obtaining the metal chelates. The detector used is connected to the sensing microzone (A membrane, B ion-exchange resin) via fibre optics in both cases. The calcium sensor [49] uses chlortetracycline, which is immobilized by adsorption onto an anion-exchange membrane that responds to Ca^{2+} ions at a neutral pH by a previously studied fluorimetric reaction [50] and transport mechanism [51].

The membrane is placed in a sandwich-shaped flow-through cell that isolates the sample on one side from optical fibres connected to the optical system depicted in Fig. 5.17.A. The sensor developers assign it a reversible character since passage of a buffer through it restores the baseline because the fluorescent chelate is very weakly retained by the membrane—in fact, some immobilized reagent is consumed, so the sensitivity decreases by 70% after a few determinations. The measurement error ($\pm 10 \text{ mM Ca}^{2+}$) is determined by fluctuations in the background signal. In any case, the most serious pitfall of this sensor lies in the fact that other cations including Mg^{2+} , Zn^{2+} and Sr^{2+} react similarly with the immobilized reagent, which is probably why it was not used for the determination of calcium in real samples.

The flow-through sensor shown in Fig. 5.17.B, developed for the determination of aluminium in real samples (dialysis fluids and concentrates), is

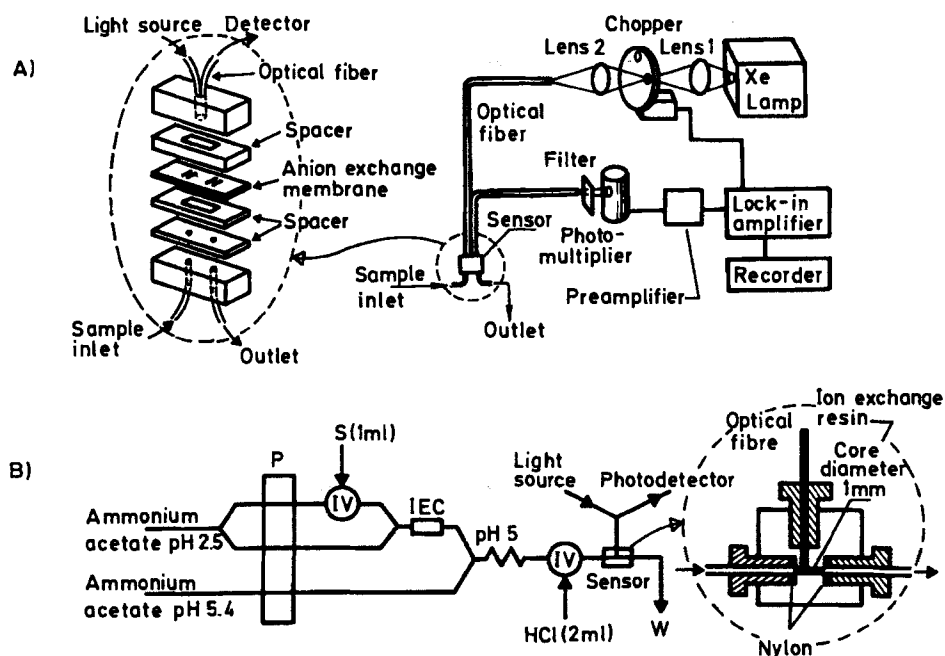


Figure 5.17 — Fluorimetric flow-through sensors for the determination of Ca^{2+} (A) and Al^{3+} (B) based on immobilization of the reagent in a membrane (A) or on an ion-exchange resin (B). S sample; IER ion-exchange reactor; IV injection valves; P peristaltic pump; W waste. For details, see text. (Reproduced from [47] and [51] with permission of Elsevier Science Publishers and the Royal Society of Chemistry, respectively).

in principle liable to pose the same problems arising from a lack of selectivity as the immobilized reagent (Kelex-100, a lipophilic derivative of 8-hydroxyquinoline), adsorbed in Amberlite XAD-7 resin, is highly reactive (even in immobilized form on solid supports [52]). Sanz-Medel *et al.* [53] provided a brilliant solution to the problem by including a mini-reactor of the same material as the sensing microzone through which the injected sample was passed at pH 2.5 in order to retain those cations forming fluorescent chelates with the reagent—aluminium obviously excluded. They also used a stream of pH 5.4 to raise the sample pH to 5 for reaction of the analyte. The sensor is of the irreversible-reusable type inasmuch as baseline restoration requires injection of 2 mL of HCl. The linear determination range achieved was 0.035–1 $\mu\text{g Al/L}$ and the detection limit 7 ng/mL. The precision as RSD was 0.8% for an aluminium concentration of 0.1 $\mu\text{g/mL}$. The most critical shortcoming of this sensor arises from interferents building up after a few determinations and requiring removal by injecting 2 mL of HCl via the sample valve after every 6–8 injections.

Several reflectance flow-through sensors for the determination of metal ions based on chelate formation in a sensing microzone accommodating the immobilized reagent have also been reported. Thus, Oliveira *et al.* [54] developed a flow-through optosensor for the determination of lead over the range 1×10^{-7} – 3×10^{-5} M with a detection limit of 1×10^{-8} M and an RSD of 8%. The light beam impinges normal to the axis of the cylindrical flow-cell used (4 mm long \times 3 mm ID) via a bifurcated fibre-optic bundle. Light reflected from the reagent phase held in the cell is also collected by means of optical fibres and guided to the detection system (a monochromator plus photomultiplier). The sensing microzone consists of a non-ionic resin (Amberlite XAD-4) onto which dithizone is previously immobilized by adsorption. The sensor is regenerated immediately after the sample stream is passed by sequentially circulating two solutions of hydrochloric acid and citrate/hydroxylamine. Neither the sensor selectivity nor its applicability to real samples were checked. Cámara *et al.* [55] reported a reflectance flow-through sensor for the determination of aluminium at concentrations from 0.25 to 1 mg/mL with an RSD of 3.6%; the configuration used for this purpose was similar to that depicted in Fig. 5.12.B. The reagent phase included in the sensor was prepared by immobilizing the chromogenic ligand (Chromazurol S) through adsorption in Dowex 1-X-2 exchange resin. A comprehensive study showed the sensor to provide acceptable selectivity

except for beryllium, phosphide, phosphate and EDTA, which interfered seriously with the determination.

Calcium can be determined at concentrations of 3–30 mM by using an optical-fibre reflectance flow-through sensor based on non-covalently immobilized calcein (2,8,8'-trihydroxy-1,1'-azonaphthalene-3,3',6,6'-tetra-sulphonic acid) on a porous anion-exchange polymer film (pore size ≥ 10 μm ; thickness 800 μm) containing *ca.* 1.5 meq/g of quaternary ammonium cation-exchange sites. The sensor is depicted in Fig. 5.18. The ten optical fibres used are sealed into slotted Plexiglass cylinders and mounted onto the cell. The solution cavity is bound by the cell front window, the silicone gasket and the thin film sensor. The colour change of the ligand in the presence of calcium is monitored at 670 nm by using the signal at 556 nm (isobestic point) as reference. A xenon lamp/optical fibres/monochromator/photometer sequence is used for this purpose. The sensor features a fairly short equilibration time (less than 15 s), a high selectivity, and compatibility

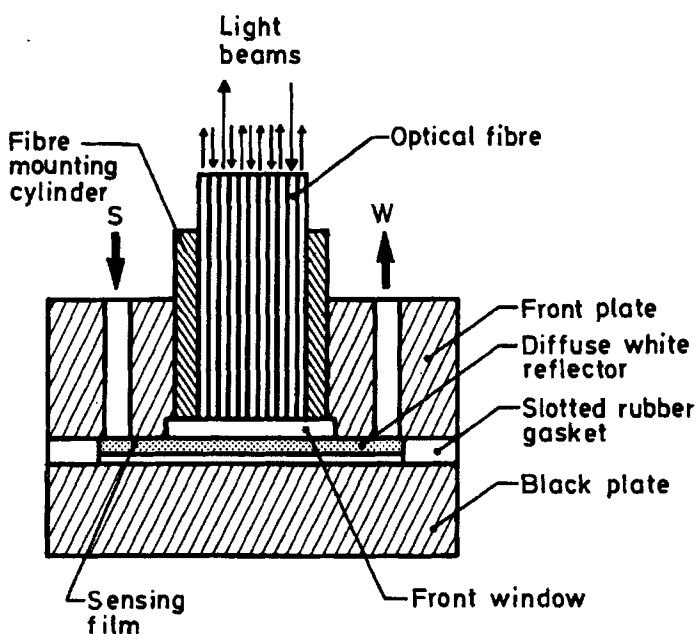


Figure 5.18 — Reflectance flow-through sensor for the determination of calcium using immobilized calcein. For details, see text. (Reproduced from [56] with permission of the American Chemical Society).

with both continuous-flow configurations and commercially available spectroscopic instruments [56].

Ruzicka *et al.* [57,58] developed some peculiar reflectance flow-through biosensors based on a sensing microzone accommodating an enzyme and an acid-base indicator (both in immobilized form) where spacial and temporal resolution of the biochemical and chemical reaction or the reversible separation of hydrogen ions was therefore impossible. The pH sensors developed by these authors (see Section 3.5.1.1) can be regarded as precedents for these reflectance sensors. The sensing approach used relies on

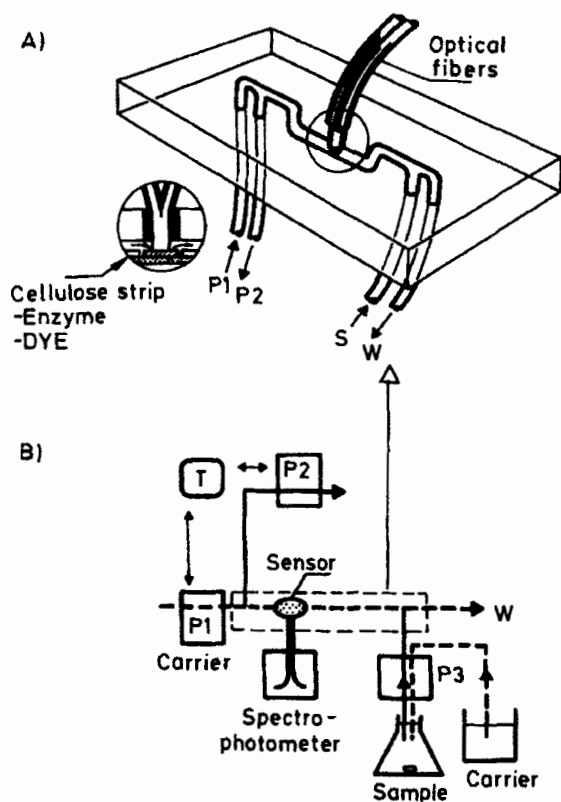


Figure 5.19 — Flow-through biochemical sensor based on the twofold immobilization of the catalyst (urease) and reagent (an acid-base azo dye) in the sensing microzone for the determination of urea in kidney dialysate. (A) Sensing microzone held in a microcircuit. (B) Valveless flow injection manifold. P pumps; T timer; S sample; W waste. For details, see text. (Reproduced from [57] with permission of Elsevier Science Publishers).

enzymatic degradation of the analyte, which results in a pH change that is detected optically by means of a coloured acid–base dye. The integrated microconduit manifold shown in Fig. 5.19.A was designed to contain a solid support (cellulose) onto which the enzyme and indicator were co-immobilized [61]. Changes in the solution pH resulted in colour changes in the sensing microzone that were monitored by reflectance measurements made with the aid of a fibre-optic bundle. The sensing microzone was readily accessible for replacement. Also, dispersion in the system was minimal, which allowed very small sample volumes to be processed. These reversible sensors are highly responsive.

Urea in kidney dialysate can be determined by immobilizing urease (via silylation or with glutaraldehyde as binder) on commercially available acid–base cellulose pads; the process has to be modified slightly in order not to alter the dye contained in the pads [57]. The stopped-flow technique assures the required sensitivity for the enzymatic reaction, which takes 30–60 s. Synchronization of the peristaltic pumps P1 and P2 in the valveless impulse–response flow injection manifold depicted in Fig. 5.19.B by means of a timer enables kinetic measurements [62]. Following a comprehensive study of the effect of hydrodynamic and (bio)chemical variables, the sensor was optimized for monitoring urea in real biological samples. A similar system was used for the determination of penicillin by penicillinase-catalysed hydrolysis. The enzyme was immobilized on acid–base cellulose strips via bovine serum albumin similarly as in enzyme electrodes [63], even though the above-described procedure would have been equally effective.

Some multiple reflecting optical waveguide flow-through sensors using reagents to retain the analytes in order to induce an optical change have also been reported [64,65]. Figure 5.20 shows them schematically. The relative humidity of air is relevant to many atmospheric processes, so there is a need for fast systems for monitoring the moisture content of air. Electronic sensors based on resistivity, capacitance and conductivity measurements are strongly affected by the temperature, whereas optical sensors, based on colour changes in metal (Co^{2+} , Cu^{2+} , V^{5+}) salts are more selective and temperature-independent as they retain water. Thus, anhydrous CoCl_2 is blue and exhibits a strong absorption peak between 600 and 750 nm; on hydration, the absorption peak shifts to 500 nm, which is characteristic of the $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ complex. This behaviour is fully reversible and lends itself readily to exploitation by use of a portable optical-waveguide humidity detector (Fig. 5.20.A) consisting of (a) a waveguide coated with a cobalt chloride/

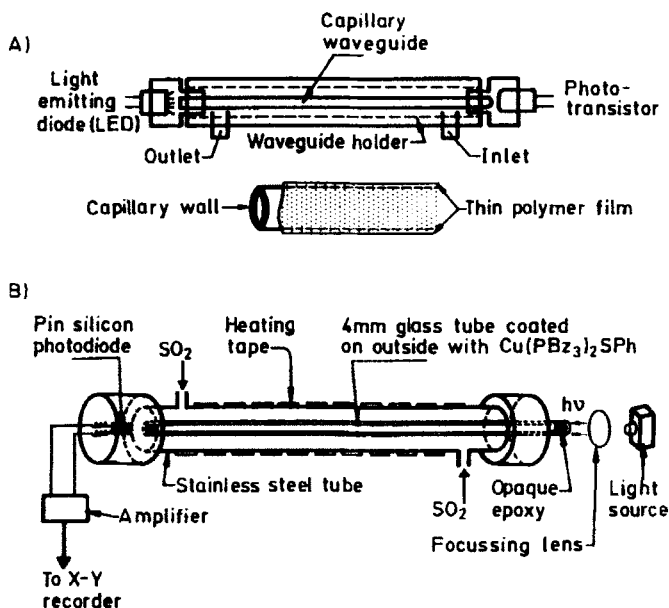
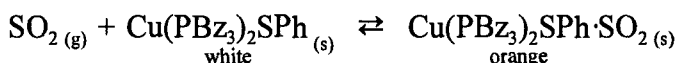


Figure 5.20 — Reversible flow-through optical waveguide gas sensors using a reagent immobilized on the inner walls of the tube for the determination of moisture (water) (A) and sulphur dioxide (B). For details, see text. (Reproduced from [64] and [65] with permission of the American Chemical Society and Elsevier Science Publishers, respectively).

poly(vinylpyrrolidone) film; (b) a straightforward, inexpensive small light source (a light emitting diode, LED); and (c) a small, affordable photo-detector (a phototransistor) [64]. The spectral response of the phototransistor drops off dramatically below 600 nm, so only the red (660 nm) and orange LEDs (635 nm) can be used as light sources. The waveguide consists of a thin-wall glass capillary tube (90 mm long \times ca. 1.0 mm OD, 0.2-mm thick wall). One end of the tube is rounded off to aid focussing transmitted light onto the phototransistor. Optical couplers made from clear plastic rods are bored to accommodate the individual components. These flow-through sensors are quite sensitive over the relative humidity range 60–95%. Data fit an S-shaped curve and are consistent with the results provided by a fibre-optic sensor [66]. In addition, this sensor design has the advantage that it can be used in field applications.

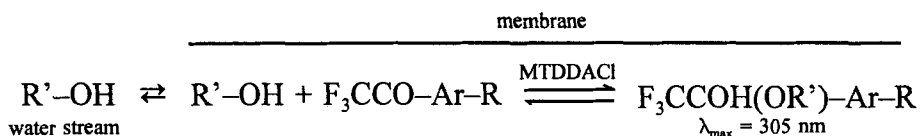
Bringing the complex formed between copper, tribenzylphosphine (PBz) and thiophenolate (SPH) in the solid state into contact with a gas containing

as little as 1 mg SO₂/L has been shown to give rise to an orange adduct resulting from retention of the sulphur dioxide in a fully reversible heterogeneous equilibrium [67]:

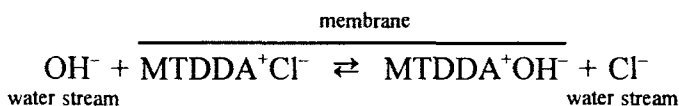


This reaction allowed the development of a flow-through optical waveguide sensor for the determination of SO₂ [65]; the same methodological principle, however, had previously been used to construct a surface acoustic wave sensor [68]. The sensor design is depicted in Fig. 5.20.B. The optical waveguide was fabricated from end-polished Corning 7740 Pyrex glass tubing (100 mm long, 4 mm OD, 0.8 mm wall thickness, 1.474 refractive index). The inside of the tube was filled with opaque epoxy resin in order to avoid direct illumination of the photodetector by light travelling down the tube axis. The outside wall was first cleaned with a phosphate buffer and the copper complex was then spray-coated onto the outside of the Pyrex tube from a 50 µg/mL solution in chloroform. A tungsten-halogen light source was used, the light beam of which was focussed on one of the tube ends, the other holding the photodetector. The sensor response was linear between 100 and 500 µg SO₂/mL (in N₂) at 60°C, and the response time was 5 min. The sensor is fully reversible, so it entails no regeneration. Neither its selectivity nor its applicability to real samples was stated, though.

Ethanol in alcoholic drinks can be determined by using a reversible photometric flow-through sensor based on an optrode membrane for retaining the derivatizing reagent [69]. This sensing approach is quite simple, rapid and inexpensive compared to methods based on distillation and density or refractive index measurements [70], and even chromatographic methods [71]. The PVC membrane used accommodates two reagents, viz. N-acetyl-N-dodecyl-4-trifluoroacetylaniline (ETH6022) and methyltridodecylammonium chloride (MTDDACl). Ethanol is extracted from the sample solution that is passed through the membrane and reacts with the carbonyl group of the reagent in a base-catalysed reaction:



The catalyst is actually hydroxyl ion, which crosses the membrane by an ion-exchange mechanism on being brought into contact with the aqueous solution



The alkaline environment inside the membrane is essential to ensure a fast, reversible response. The sensor is similar to that shown in Fig. 5.6 (the gas-diffusion membrane excluded). Two identical membranes of *ca.* 4 μm thickness are coated on two quartz-glass plates by means of spin-on device [72] and then mounted in the flow-cell [10]. The membranes are conditioned by immersion in distilled water for a few minutes prior to use. Photometric measurements are made on a dual-beam spectrophotometer furnished with a reference cell of the same shape as the measuring cell but containing no membrane. Ethanol can thus be determined at concentrations from 0.5 to 35% v/v in aqueous solutions. In addition to a high reproducibility in the optical signals, the system features a response time of less than 30 s. The sensor exhibits a preference for ethanol over water, isopropyl alcohol and *t*-butanol by a factor greater than 10, and a similar response to other primary alcohols including methanol, 1-propanol and *n*-butanol. The results obtained by applying the sensor to the determination of the potency of various alcoholic drinks (wines, beers and liqueurs) were quite consistent with those provided by the conventional distillation/density measurement method. A residual standard deviation of $\pm 0.27\%$ v/v was obtained over the concentration range 0.7–40% v/v ethanol.

Cardwell *et al.* [73] reported a photometric flow-through sensor using an acid-based indicator immobilized on a cellulose membrane prepared *in situ* that contained an ion-balance reagent (sodium tetraphenylborate). This additive ensured compatibility between the reagent and membrane, thereby avoiding reagent losses on passage of the flow. In addition, the ion-balance reagent resulted in a much more rapid response relative to other sensors [74]. The membrane was deposited by evaporation of a mixture of its constituents (cellulose acetate, diethyl phthalate, an azo dye of $pK = 3.8$ and sodium tetraphenylborate) in tetrahydrofuran on the wall of a 14 mm \times 2.3 mm ID tunnel roughened with a diamond drill for improved adhesion to the membrane in a 7.5 mm OD glass cylinder which in fact acted as the sensor.

The whole assembly was placed in a discontinuous flow analyser [75] for the titration of 0.1 M NaOH with 0.1 M HCl.

A flow-through sensor based on twofold (electrochemical and mass) detection was recently developed for the determination of electroinactive cations (*e.g.* K^+) in aqueous solutions [76]. The sensor was constructed by depositing a film of Prussian Blue (PB, an electroactive mixed-valence iron–cyanide complex) on a quartz crystal microbalance (QCM) coupled on-line to a flow injection manifold but still usable batchwise for other applications. During the reversible reduction–oxidation of PB, cation transfer in and out of the film was found to prevail over anion transfer —hence the electrochemistry of PB films was affected by the type and concentration of cations in solution. In order to make detection specific, the mass of cations in the film was measured during the voltammetric response by means of the QCM, the frequency of which should reflect both such a mass and that of solvent transferred in and out of the film, which had previously been obtained in isolation [77]. The microbalance used AT-cut quartz wafers of 2.5 cm diameter with fundamental frequencies close to 0.5 MHz. The electrode pads, consisting of a 2.5-mm thick Cr film over a 1.5-mm thick Au film, were evaporated onto the quartz wafers [78,79]. Prussian Blue films were deposited onto the working electrode by electroreductive crystallization from a solution containing 2 mM $FeCl_3$ and 2 mM $K_2Fe(CN)_6$ adjusted to pH 2 with HCl. Use of this dual-purpose sensor in a flow system affords expeditious sample and concentration changes. In fact, by having the PB film on the QCM under potentiostatic control, it responds to changes in the cation type and concentration in two ways, *viz.* (a) by passing a redox current, and (b) by changing the frequency. In this way, a twofold response (a current density in $\mu A/cm^2$ and a frequency in Hz) is obtained from potassium ion.

5.4.2.2 Ionophore optical sensors

Neutral ionophores, which were formerly widely used in the development of selective electroanalytical sensors [80–84], have lately started to be incorporated into optical sensors. These species facilitate ion exchange between a solution containing the analytes (cations, anions or even ionizable neutral species) and the components of a lipophilic phase, which undergo an optical (colour of fluorescence) change that is sensed by the detector employed (usually integrated with the sensing microzone). In fact, neutral ionophores act as ion carriers forming complexes or charged associates (usually of 1:1 stoichiometry) with the "extracted" analytes. This reversible

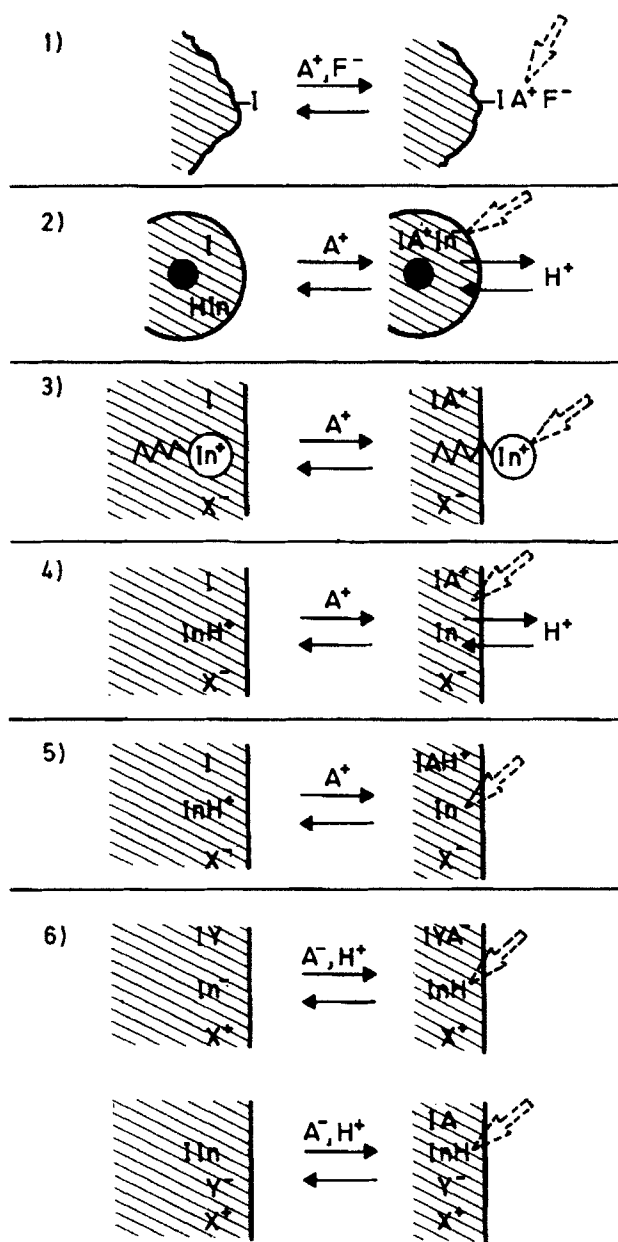


Figure 5.21 — Mechanisms involving an optical change in flow-through metal ionophore-based sensors. The ionophore (I), indicator (In) and hydrophobic counter-ion (X) are in the lipophilic phase (shaded area), which can be a solid (1), a layer (2) or a membrane (3–6). The analyte (A) can be a cation (1–4), an anion (6) or a neutral species (5). The dotted arrow indicates the origin of the optical change, which is always related to the indicator. For details, see text.

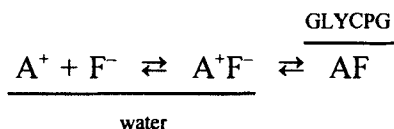
mass transfer process may be accompanied by the release of a hydrophilic charged species in the lipophilic phase (*e.g.* a proton). As the analyte is introduced into this phase, the lipophilic indicator it contains undergoes a reversible chemical change that in turn produces an optical change. Preserving the electroneutrality of the organic phase, which can be a membrane or a solid covered with a lipophilic coat, usually entails incorporation of a lipophilic anionic or cationic species. As a rule, the reagents contained in the organic phase are of lipophilic nature (hydrophilic substances must previously be modified chemically by introducing long aliphatic hydrocarbon chains).

The practical interest of this optical sensing approach lies in (*a*) the reversibility of the sensing process involved; (*b*) a fairly high sensor responsiveness; (*c*) the selectivity provided by a neutral ionophore reacting virtually specifically with the analyte; (*d*) its suitability for flow measurements. It is interesting to note that most of these optrodes have been used in flow-through sensor applications, which is indicative of the advantages involved. However, few of the reported uses of these flow-through sensors based on a triply integrated process (see Fig. 5.1.C) include an on-line coupled continuous-flow configuration. No space or time resolution of separation, reaction and detection is possible as all three take place virtually simultaneously at the sensing microzone.

Figure 5.21 illustrates some of the more common response mechanisms associated with ionophore-based optical flow-through sensors. Mass transfer in these sensors relies on association of the ionophore with the analyte (IA), which is incorporated into the organic phase (occasionally from the aqueous phase). The transfer may involve a single species (the analyte, Figs 5.21.1–5) or two (the analyte plus another ion, Fig. 5.21.6). Also, this ionic separation may involve straightforward retention (Figs 5.21.1, 5.21.3, 5.21.5 and 5.21.6) or ion exchange by transfer of an ion of the same sign in the opposite direction (Figs 5.21.2 and 5.21.4). The indicator may be in the same aqueous solution as the analyte (Fig. 5.21.1) or lipophilic and initially present in a neutral (Fig. 5.21.2), charged acid (Fig. 5.21.4 and 5.21.5), cationic (Fig. 5.21.3) or anionic form (Fig. 5.21.6) in the lipophilic phase. The colour of fluorescence change observed arises from the formation of an ion-pair that concentrates the indicator (Fig. 5.21.1), a moiety change (Fig. 5.21.3) or a typical acid–base process (Figs 5.21.2, 5.21.4, 5.21.5 and 5.21.6). All the mechanisms shown in Fig. 5.21 are reversible to some extent, *i.e.* changes in the aqueous solution in contact with the organic sensing microzone result

in changes in the signal provided by the sensor. If the time needed to reach the equilibria involved in the sensing process (mass transfers between the two phases) is fairly long, the sensors are apparently irreversible. Below are described some of the more significant optrodes using neutral ionophores, developed by the research groups headed by such renowned authors as Seitz, Wolfbeis, and the late Ishibashi and Simon.

Seitz *et al.* [85] developed a chemical flow-through sensor based on the non-covalent immobilization of a neutral ionophore (*e.g.* valinomycin) on particles of controlled pore glass and a fluorescent anionic probe (8-anilino-1-naphthalenesulphonic acid). The modified support was incorporated into a flow-cell consisting of a piece of Tygon tubing of 1/16 in. ID that was placed in an ordinary fluorimeter. The mechanism of action involved is illustrated in Fig. 5.21.1. On passage of the sample stream containing potassium (A^+) and the fluorescent probe (F^-) by the active microzone, the ionophore forms a complex with potassium, the positive charge of which allows the indicator to be retained. This concentrating effect results in increased fluorescence. When the stream contains no potassium, the signal decreases through disappearance of the complex. The ensuing optical change results from quenching of the fluorescence of the probe in the aqueous solution as opposed to a markedly enhanced fluorescence on the hydrophobic surface of the controlled pore glass. The most critical pitfall of this sensor arises from the loss of ionophore on passage of the flowing stream. Adding some ionophore to the stream is no solution because of its low water-solubility and high cost. Glyceryl controlled pore glass (GLYCPG) beads containing no ionophore also give a fluorescence signal proportional to the cation concentration but entail liquid-liquid extraction of the ion-pair formed in the flowing stream:



The selectivity thus achieved is much lower than that provided by the ionophore; however, the system features a shorter response time and can be used for a virtually unlimited time with no losses. It can readily be adapted for the determination of K^+ , Na^+ and Ca^{2+} —the last ion can be highly selectively determined in the presence of magnesium.

Ishibashi *et al.* [86] reported a fluorimetric flow-through sensor based on the mechanism schematized in Fig. 5.21.3 which they had previously used to implement probe-type sensors [87,88]. Essentially, the mechanism involves formation of a complex (IA^+) by transfer of the analyte (potassium ion, A^+) to an organic phase on bringing it into contact with a PVC membrane containing a neutral ionophore (valinomycin), a counter-ion [3,5-bis(trifluoromethyl)phenylborate] and a fluorescent cationic indicator with a lipophilic chain (Hexadecylacridine Orange). In order to preserve the electroneutrality balance, part of the cationic indicator was transferred to the aqueous phase (the lipophilic chain ensures retention by the membrane). This response mechanism is similar to Waggoner's on-off mechanism [89]. The optical change observed is due to the fluorescence quenching arising from passage to the aqueous environment and is proportional to the potassium concentration. Because the system was reversible, the fluorescence baseline was restored as the analyte lost contact with the membrane. Figure 5.22 depicts the sensing microzone as integrated with an ordinary spectrofluorimeter (A) and the on-line FIA manifold used with it (B). The PVC membrane was deposited by conventional evaporation on one of the sides of the flow-cell (12 μ L inner volume). To its initial ingredients (I and

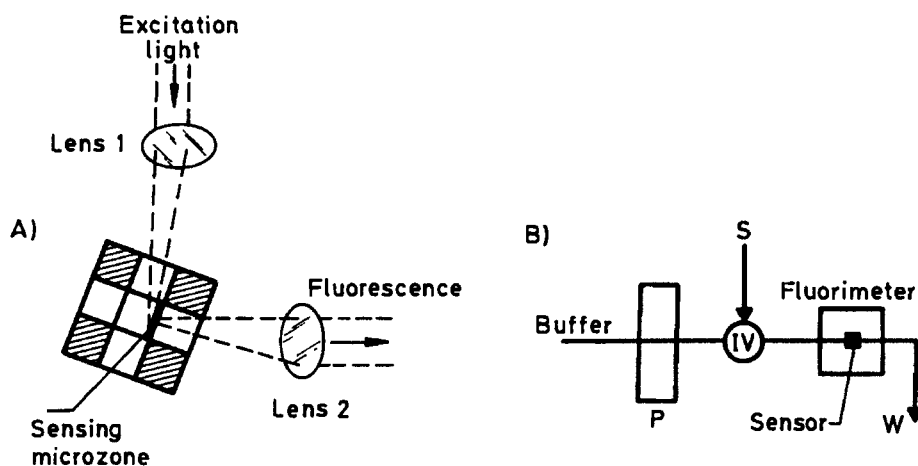
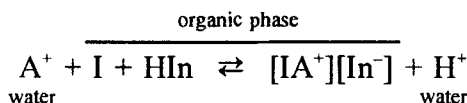


Figure 5.22 — Reversible flow-through fluorimetric sensor for the determination of potassium in human blood plasma based on the mechanism shown in Fig. 5.21.3. (A) Flow-cell containing the lipophilic membrane. (B) Flow injection configuration. P pump; IV injection valve; W waste. For details, see text. (Reproduced from [86] with permission of Elsevier Science Publishers).

X⁻Na⁺) the indicator was added by ion exchange with sodium, displacement of the equilibrium to the indicator being favoured by its hydrophobicity. The single-channel continuous-flow manifold used included a peristaltic pump and an injection valve (100-μL sample loop) through which a buffer stream at pH 7.5 was circulated. The linear response range for K⁺ was 0.5–50 mM and the peak height was proportional to the logarithm of the ion concentration. The throughput was 15 samples/h and the RSD 2.4% for 1 mM potassium. Sodium was found to introduce a positive error in the determination (2% and 12% increase in the negative peak height for 10 and 100 mM Na⁺ in the presence of 1 mM K⁺); accordingly, potassium could be determined in human blood, which typically contains *ca.* 3.8 mM potassium and 140 mM sodium, with an error of 3.3% (3.0% expected). The authors carried out an interesting study of the influence of the type of lipophilic chain borne by the fluorescent indicator and its length.

Suzuki *et al.* [90] developed a diffuse reflectance flow-through sensor for improving selectivity in the determination of lithium in human fluids, where sodium can occur at concentrations up to 10 000 times higher than that of the analyte. The mechanism on which the sensor action relies (Fig. 5.21.2) was previously exploited by the authors to develop a probe-type sensor for potassium [91]. The sensor response is based on an A⁺–H⁺ exchange according to



where A⁺ denotes the analyte (Li⁺), I the neutral ionophore (a new 14-crown-4 derivative) and HIn the lipophilic dye [4-dinitro-6-(octadecyloxy)phenyl-2',4'-dinitro-6'-(trifluoromethyl)phenylamine, LAD-3, N-2]. Figure 5.23.A shows the three mass transfer equilibria and two chemical equilibria that take place between the sensing microzone and the solution in contact with it. The microzone consists of pellicular octadecylsilane (ODS) beads onto which the ionophore and LAD-3 are incorporated from special organic solvents [*bis*(2-ethylhexyl)sebacate and *o*-trifluoromethylphenyl dodecyl ether]. The structure of this fibre-optic flow-through sensor is depicted in Fig. 5.23.B. The lithium sensing beads are placed on a mirror and packed in a 7-μL compartment including a quartz window that is attached directly to the tip of a bifurcated optical fibre to perform diffuse reflectance measurements. The mirror has a hydrophobic surface modified by silylation in order to prevent the beads

from being carried away from the cell. A similar cell filled with ODS beads holding no dye is used as reference. Both cells are connected to the same light source, furnished with a monochromator, and a double-beam spectrophotometer (monochromator plus detector). The on-line coupled manifold used for implementation of this sensor comprises a pulse-free pump and an HPLC injection valve furnished with a 2-mL sample loop and crossed by a single buffer stream. The sensor can detect at Li^+ concentrations from 10^{-6}

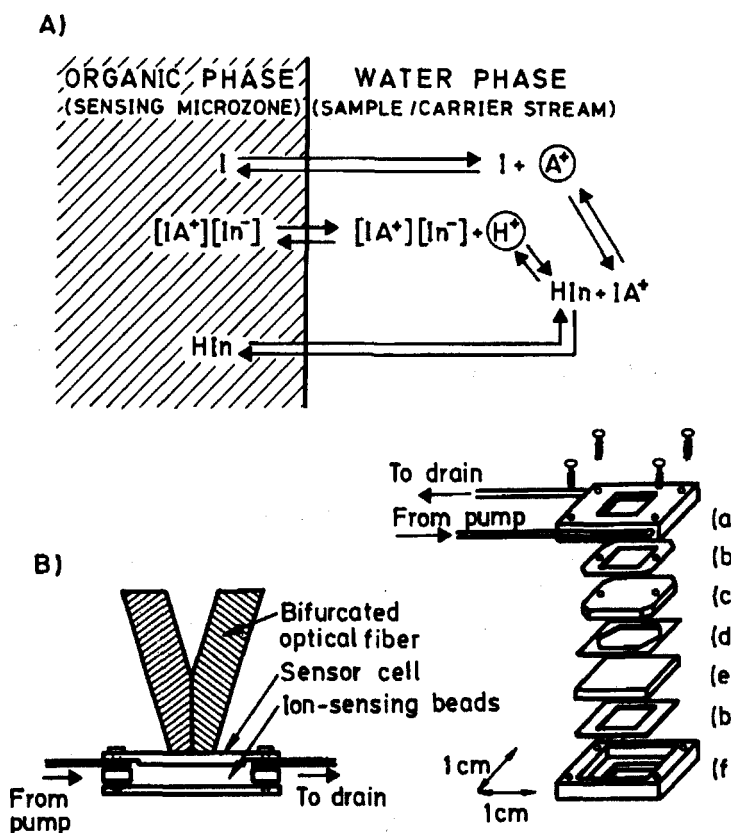


Figure 5.23 — Flow-through ionophore-based sensor for the determination of lithium in serum. (A) Mechanism involved in the sensor response (symbol meanings as in Fig. 5.20). (B) Diffuse reflectance flow-cell: (a) upper stainless steel cell body; (b) silicon rubber packing; (c) quartz glass window; (d) Teflon spacer (0.05 mm thickness); (e) hydrophobic surface mirror; (f) lower stainless steel cell body. For details, see text. (Reproduced from [90] with permission of the American Chemical Society).

to 10^{-1} M at pH 7. The response time is *ca.* 60–100 s, but baseline restoration takes 15–20 min. This flow-through optrode features excellent selectivity towards Li^+ ion (factors of over 10^4 relative to all alkaline and alkaline-earth metal cations) and allows the determination of millimolar levels of the analyte in serum samples from manic depressive patients.

Table 5.1. Simon-type ionophore based flow-through sensors

Analyte	Mechanism	Real sample	Reference
NH_4^+	Fig. 5.21.4		10
NH_3 (NH_4^+)	Fig. 5.21.5		9
Ca^{2+}	Fig. 5.21.4		94
Pb^{2+}	Fig. 5.21.4	Waters ^(a)	96
Na^+	Fig. 5.21.4	Serum	97,99
K^+ ^(b)	Fig. 5.21.4		105
CO_3^{2-}			102
NO_3^- ^(c)	Fig. 5.21.6	Vegetable extracts	101
Cl^-	Fig. 5.21.6	Serum	100
Enantiomers ^(b)	Fig. 5.21.4		104

^(a) Possible, but not implemented

^(b) Fluorimetric (the others are photometric)

^(c) Probe-type ISPD (ion-selective photodiode)

Table 5.1 lists the main types of optical flow-through sensors based on the use of neutral ionophores, most of which were developed by Simon's group. Their mechanisms of action are illustrated in Figs 5.21.4 and 5.21.6, and were discussed elsewhere [92,93]. These sensors have been applied to both cationic, anionic and neutral-ionizable species. All rely on a (usually) colour change undergone by an acid–base indicator referred to as an " H^+ ion-selective chromoionophore" that can be neutral (Figs 5.21.4 and 5.21.5) or anionic (Fig. 5.21.6). The mechanism may involve exchange of a proton with the analyte, as well as co-extraction. The flow-cell used in every case is similar to that in Fig. 5.6, so these sensors are of the integrated rather than the connected type (see Fig. 2.6.C). The sensing microzone is a plasticized PVC membrane of 0.5–2.0 μm thickness that is attached to the cell glass or quartz window by using a spinning device furnished with a closed aluminium/Plexiglass cell at a rotating frequency of 600 min^{-1} [70]. Few of these sensors have been used in on-line connections to continuous-flow manifolds.

The flow-through sensors of this type for ammonium ion [10] and ammonia [9] differ in the presence of a gas-permeable membrane parallel to the sensing PVC membrane and containing (a) a cation-selective neutral ionophore (I, nonactin, monacin, donactin, tetranactin or valinomycin); (b) Nile Blue-line H^+ ion-selective neutral dyes, which are highly basic and hydrophobic; (c) a lipophilic anion [potassium *tetrakis*(*p*-chlorophenyl) borate]; and (d) a plasticizer [*bis*(2-ethylhexyl)sebacate]. The mechanisms of action involved are different for the two species and depend on the original charge of the analyte (see the scheme in Fig. 5.21.3 for NH_4^+ and that of Fig. 5.21.4 for NH_3 transferred across the gas-diffusion membrane as shown in Fig. 5.6). The absorbance was found to be linearly related to the NH_4^+ activity over the range 10^{-5} – 10^{-3} M at pH 7.35. The selectivity achieved was similar to the potentiometric selectivity of ion-selective electrodes based on a neutral ionophore (nonactin or monacin) [10]. The determination of ammonia by use of this type of sensor was discussed in Section 5.2.1.

Simon *et al.* [94] developed a flow-through optrode for the determination of Ca^{2+} based on the same principle as the above-described ammonium ion sensor (Fig. 5.21.4). The sensor used a classical ionophore for calcium that was previously employed to construct selective electrodes for this ion [95] and a new H^+ ion-selective dye (a lipophilic isologue of Nile Blue) containing lipophilic anionic sites), both held in the same plasticized PVC membrane. The sensor configuration was identical with that of the previous sensor. The high selectivity of the ionophore and the reversible sensing approach used resulted in interesting analytical features such as a wide linear response range (from 10^{-5} to 10^{-2} M Ca^{2+}), good precision ($\text{RSD} < 1.5\%$), a response time of a few seconds, and adequate selectivity. These authors assayed various neutral ionophores and H^+ ion-selective chromoionophores (dyes) in order to develop a sensor for the determination of lead over a wide concentration range (5×10^{-9} – 5×10^{-3} M) with good sensitivity (detection limit 3.2×10^{-12} M) [96]. The best results in terms of sensitivity were provided by the ionophore ETH-332 (previously used to construct ion-selective electrodes), even though the selectivity towards alkaline-earth metals was somewhat degraded. Other ionophores tested (*e.g.* ETH-5428) resulted in improved selectivity but lacked the sensitivity required for application of the flow-through sensor to the determination of lead in environmental samples below legally established levels.

The sample pH plays a major role in the operation of all Simon-type sensors since protons are involved in their response mechanism and affect not only their sensitivity but also their selectivity. In acknowledgement of this fact, Simon *et al.* [97] developed a novel selectivity formalism based on the different equilibria involved in the sensor responses in the absence and presence of foreign ions. Such a formalism assumes the chromo-ionophore to be ideally proton-selective. At a constant pH, a selectivity coefficient is given by the activity ratio of the analyte to the interfering ion, which lead, on separate calibration, to the same degree of protonation of the chromoionophore. This innovative theory is especially helpful with ions of different valences, for which no appropriate description had so far been provided. Simon's definition for the selectivity coefficient differs from that in the Nicolsky–Eisenman formalism [98] and is independent of the sample pH. Simon's theory was used to calculate the calcium selectivity of a flow-through sensor for sodium based on the ionophore ETH-4120, which turned out to be consistent with the experimental value (the coefficient is high enough for calcium to be determined in serum with no interference from sodium) [99].

Determinations of anionic species by use of Simon-type flow-through sensors rely on the simultaneous co-extraction of the counter-ions (*e.g.* Cl^-) and the protons in the aqueous solution, which are incorporated into a PVC membrane by interaction with the ionophore (trioctyltin chloride, R_3SnCl) and an anionic lipophilic indicator [fluorescein(2-octyleicosyl)ester], respectively. The membrane also contains a quaternary ammonium ion. Figure 2.21.6 shows the two possible mechanisms of action derived from the neutral nature of the ionophore (IY). Research in progress involving ^{119}Sn -NMR spectra is aimed at distinguishing between the two processes [100]. This chloride ion sensor provides a linear response between 10^{-1} and 10^{-3} M Cl^- at pH 3.6 using 0.1 M formic acid as buffer. Stable readings can be obtained within *ca.* 3 min after flushing the sensor with each sample solution. The RSD achieved ranges from 1.6% to 2%. Application of the sensor to the determination of chloride ion in serum provided results that were essentially consistent with those obtained by using a Hitachi 737 autoanalyser based on potentiometric (ISE) measurements. This sensing approach was also applied to the determination of nitrate ion in vegetable extracts [101] by replacing the flow-cell with a simple probe including a couple of photodiodes.

Based on an optrode previously reported by Simon *et al.* for the determination of 1-phenylethylammonium ion using a crown ether ionophore

[103], Wolfbeis *et al.* [104] developed a fluorimetric flow-through sensor for resolving protonated enantiomers of 1-phenylethylamine, propanolol and norephedrine. The sensor used (R,R')tartrates (lipophilic esters of tartaric acid) as ion-carriers (ionophores). The mechanism of action of the sensor is illustrated in Fig. 5.21.4. The flow-cell used to house the PVC lipid membrane [105] was designed for fluorimetric rather than photometric measurements, as usual in Simon's work. The membrane also accommodated a lipophilic pH fluorimetric indicator (DZ-49) and a lipophilic anion [*tetrakis*(4-chlorophenyl)borate]. The sensor proponents determined both free energy differences between the two enantiomeric complexes and the selectivity coefficients for the enantiomers from calibration graphs. Figure 5.24 shows the response curve (A) and the Stern–Volmer calibration plot (B) run for the two enantiomers of 1-phenylethylamine; as can be seen this sensing approach offers an unusual resolving power for a (bio)chemical sensor. Even though the best selectivity coefficient was obtained for norephedrine using (1R,2S,5R)-dimethyl-(R,R)-tartrate as the ionophore, (R,R)-di-*tert*-butyltartrate was the most effective ionophore in all other instances. The sensor is fully reversible and features linear determination ranges from 7×10^{-3} to 7×10^{-1} mM for 1-phenylethylamine, 6×10^{-3} – 7×10^{-2} for propanolol and 0.7–7.0 mM for norephedrine at a near-neutral pH. The response time is *ca.* 2 min for a membrane thickness of approximately 1 μm .

The photometric flow-through sensors developed by Simon's group can readily be adapted for fluorimetric measurements by using two indicators in the membrane; such indicators act via an inner filter effect of fluorescence (IFE) rather than by energy transfer. One of the dyes (the absorber) changes colour with the pH and acts as an H^+ ion-selective chromoionophore, whereas the other (the fluorescer) is a pH-independent fluorophore bound to commercially available minute particles contained in the sensor membrane. The other components of the 2- μm thick membrane used are those usually found in Simon-type sensors, *viz.* (a) an ion-selective ionophore (valinomycin for determining potassium); (b) a lipophilized proton-carrying dye (an azo derivative); and (c) a lipophilized anion [*tetrakis*(4-chlorophenyl)borate]. The response mechanism of the sensor is similar to that illustrated in Fig. 5.21.4. The fluorescence of the fluorophore particles changes in response to variations in the absorption of the coloured dye, which in turn is modulated by the potassium concentration through the IFE. The sensor is fully reversible for potassium in the range from 1 μM to 10

mM and features a useful dynamic range of 1 μM to 1 mM. Its lifetime exceeds three months when stored in the dark at 4°C. When sensing aqueous samples, the response drifts by -4% over 2.5 h, seemingly as a result of photobleaching of the coloured dye. Unlike electrochemical-ISE measurements, this optical sensor is pH-dependent, which is a serious constraint in dealing with real samples.

Wolfbeis *et al.* [105–107] developed several fluorimetric flow-through sensors based on a sensing microzone consisting of a multilayer lipid membrane formed on a glass support by using the Langmuir–Blodgett (LB)

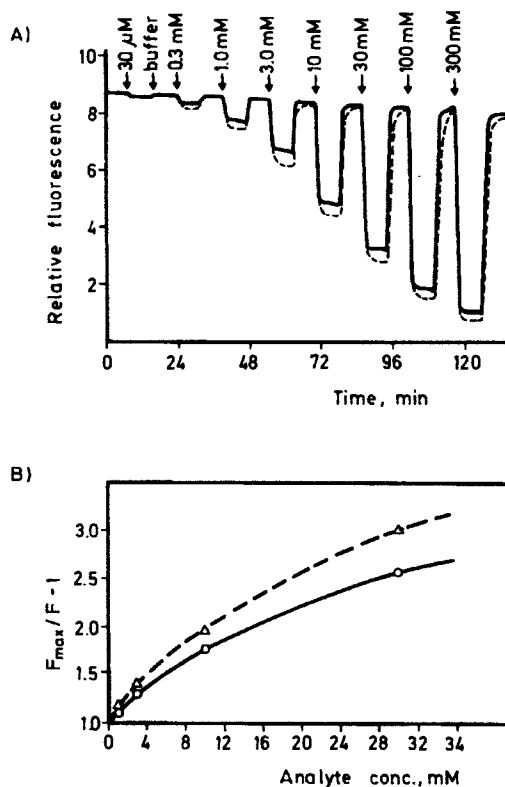


Figure 5.24 — Enantioselective fluorimetric flow-through optrode using a lipophilic tartrate (di-*tert*-butyrotartrate) as ion-carrier for the determination of (protonated) 1-phenylethylamine. (A) Sensor response to increasing concentrations of the analyte [(—) S enantiomer; (---) R enantiomer]. (B) Stern-Volmer calibration plot. F_{max} and F denote fluorescence intensities ($\lambda_{\text{exc}} = 520$, $\lambda_{\text{em}} = 600$ nm) in the absence and presence, respectively, of the analyte. (Reproduced from [104] with permission of Elsevier Science Publishers).

method. The sensors rely on the ability of some fluorescent dyes to respond to an electric potential at the lipid/water interface established on addition of a neutral ion-carrier (ionophore) to the membrane in the presence of the analyte. The net effect is a decrease in the fluorescence of the indicator, which must be made highly hydrophobic by chemical modification. The membrane formation is completed by introducing another lipophilic-polar component.

Changes in the membrane potential can arise from charge separation across the membrane caused by the ionophore, streaming potentials at the membrane surface caused by the flow of sample solution, and, possibly, the formation of diffusion potentials. These potential changes induce optical changes that may be ascribed to three different effects, namely: (a) the Stark effect (*i.e.* changes in the absorption and emission spectra of a fluorophore on application of an external field); (b) changes in the otherwise homogeneous distribution of the fluorophore within the lipid membrane on creation of an electric field leading to, for example, aggregation and self-quenching; and (c) a field-dependent distribution of the dye between regions of different polarity within the lipid membrane resulting in a solvatochromic effect. The lack of selectivity of the response arises from the also limited selectivity of the ionophore, as well as from the streaming and diffusion potentials established. The latter two can be offset by using a reference optrode, but the ionophore's lack of selectivity is insurmountable.

Potassium can be determined by using valinomycin as ionophore, the C₁₈ ester of rhodamine B as sensing dye and arachidic acid [106] or 1-octadecanol [107] as a lipidic component of a sensor membrane. The LB technique for controlled deposition of multilayer membranes is highly reliable and reproducible. The layers are formed on glass plates that are accommodated in a fluorimetric flow-cell. Figure 5.25.A shows a six-layer membrane. Membranes consisting of six or more heterogeneous layers provide a less pronounced response to potassium than do membranes with fewer layers, which are not stable enough. This shortcoming can be circumvented by preparing membranes comprising two types of layer. The uppermost layer containing the potential-sensitive dye is placed directly at the lipid/water interface, whereas the other layers, containing valinomycin only, are held between the silylated surface of the glass support and the dye-labelled layer. All three components are reportedly required for proper functioning of the potassium sensor. The best results are obtained with 1-octadecanol and valinomycin in the membrane. The sensor features a linear

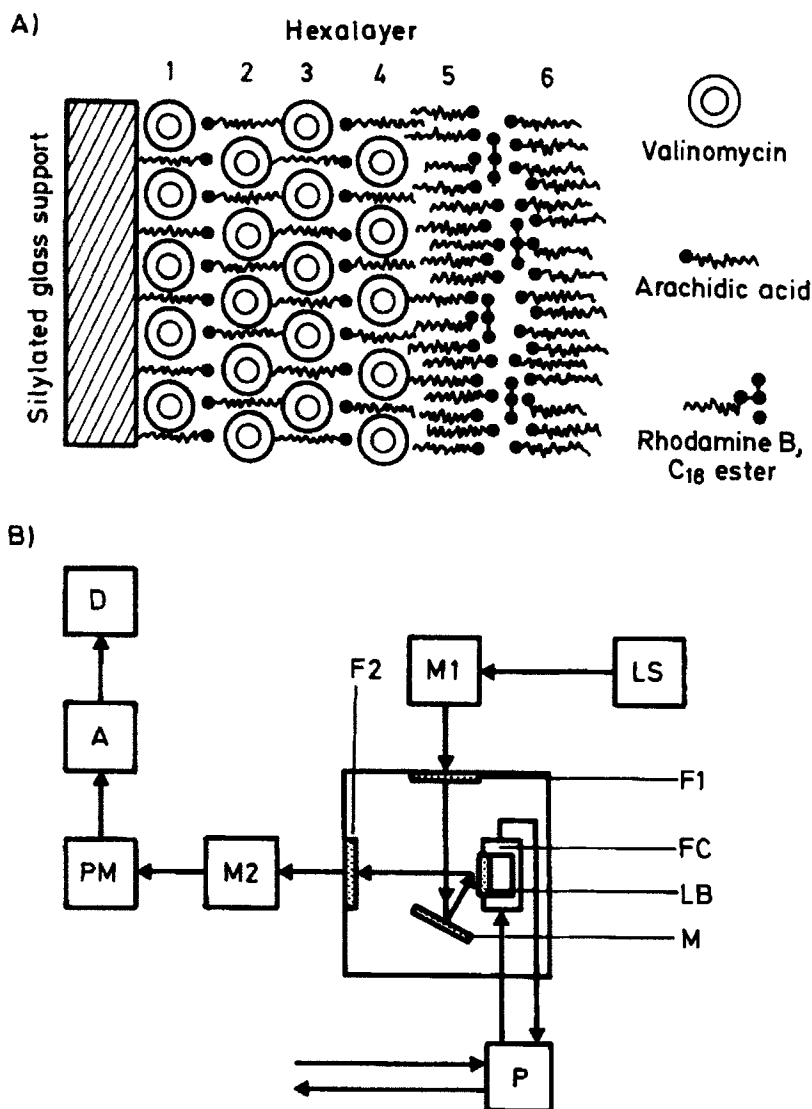


Figure 5.25 — Flow-through K^+ ion-selective optrode based on a multilayer lipidic membrane prepared by the Langmuir–Blodgett method. (A) Cross-sectional view of the composite six-layer membrane (four layers of arachidic acid/valinomycin covered by an arachidic acid and rhodamine dye bilayer). (B) Optical arrangement integrated with the sensor, which is connected to a flow system. LS light source; M1 and M2 excitation and emission monochromator, respectively; F1 and F2 primary filters; M mirror; LB lipid-sensitive membrane in a glass platelet; FC flow-cell; A amplifier; D display; P peristaltic pump. (Reproduced from [107] with permission of the Royal Society of Chemistry).

relationship between the $\Delta I/I$ ratio (ΔI denotes the variation of the fluorescence intensity and I the total intensity) and the logarithm of the potassium concentration over the range 10^{-2} – 10^2 mM. The most serious pitfall of this sensor is the poor selectivity arising from its parallel response to other metal ions (via a species other than the ionophore); in any case, the selectivity factor for sodium is 2.5–5.0 and the interference from foreign ions can be minimized by using a reference optrode. Figure 5.25.B depicts the integrated fluorimeter–sensor–flow manifold system.

A very similar approach was used to develop a fluorimetric flow-through sensor based on the Langmuir–Blodgett method for the determination of calcium. The lipid membrane was formed on a silylated glass plate by combining a lipid (1-octadecanol), a hydrophobic dye (the C_{18} ester of rhodamine B) and a commercially available neutral ionophore (ETH-1001, a crown ether) with a high selectivity towards calcium. The sensing membrane, prepared by the LB method, consisted of two layers containing the lipid, ionophore and C_{18} -Rho B. The reference membrane was made similarly from the same components except the ionophore. An "optical Nernst equation" was used to establish a relation between the negative logarithm of the calcium concentration over the range 0.1–10 mM and the decrease in the relative fluorescence. The reference optrode provided selectivity factors higher than 10^5 for Mg^{2+} , Na^+ and K^+ . The membrane was stable for six months when stored in the dark. The most critical drawbacks of these LB-based optical sensors developed by Wolfbeis' group include (a) the need for sophisticated instrumentation; (b) the imperative use of a reference sensor (containing no ionophore) to avoid interferences; (c) the small changes in the relative signal obtained; and (d) a poor signal-to-noise ratio. These shortcomings may be circumvented by using new methodological approaches involving laser excitation as well as other electrochromic dyes, polymer LB films [108] or the evanescent wave technique [109].

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